


Classical Swine Fever in Wild Hog: Report of its Prevalence in Northeast India


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Summary

Classical swine fever virus (CSFV) is the causative agent of a highly contagious disease, hog cholera in pigs. The disease is endemic in many parts of the world and vaccination is the only way to protect the animals from CSFV infection. Wild hogs belong to the species Sus Scrofa Cristatus under the family Suidae are quite susceptible to CSFV infection. The epidemiological role concerning classical swine fever (CSF) in India is largely unknown. We report here the three isolated cases of CSF in wild hogs from three National parks, namely Kaziranga National Park, Manas National Park and Jaldapara National Park, from north-east part of India. The post-mortem and histopathological findings were clearly indicative for CSFV infection. The presence of CSFV genome was demonstrated in several organs and tissues collected from hogs died due to viral infection. In addition, CSF-specific antibodies were detected in two wild hogs as well as in eighteen feral pigs from the same locations. The phylogenetic analysis of the partial E2 protein gene and 5′ untranslated region of CSFV isolates from the wild hog showed identities with genotype 2.2 of the Indian isolates. Occurrence of CSF in wild hogs may pose a potent threat in the epidemiology of the virus in Northeast part of India. To the best of our knowledge, the report presented in the manuscript is the first comprehensive report on CSF in wild hogs from Northeast India. The findings reported would help us to understand the epidemiology and biology of CSFV in wild animals.

Introduction

Classical swine fever (CSF) is an important viral disease of domestic, wild and pygmy hogs (Depner et al., 1995; Meuwissen et al., 1999; Laddomada, 2000; Kaden et al., 2004; Barman et al., 2012). Classical swine fever infection causes substantial economic losses in the countries where pig farming is a major source of livelihood (Edwards et al., 2000). Classical swine fever virus (CSFV) belongs to the genus Pestivirus in the family Flaviviridae. The disease occurs in most parts of the world including India barring North America and Australia.

Control as well as eradication of CSF in domestic pigs is a great challenge in most part of the globe as the virus can be maintained in reservoir wild hog populations and reintroduced into domestic pigs resulting in frequent outbreaks (Artois et al., 2002; Moennig et al., 2003). It is also possible that CSF can rapidly crossover from domestic animals to captive or free ranging wild animals of the closer habitats through direct contact with the infected domestic pig.
contaminated feed, water and humans trespassing. In Assam (India), large population of wild hogs is distributed in different National Sanctuaries and game forests. Human population at rural areas utilizes forest for agricultural as well as livestock rearing. Interestingly, pig farming and rearing in a free grazing or in scavenging way is a tradition of local tribal population. Moreover, in such conditions, domestic pigs share same grazing land with wild hogs. Interchanging of swine diseases, including CSF between domestic and wild population is quite possible. Studies indicated that about 77% of infectious diseases of domestic animals including CSF are common to wild life (Karesh et al., 2005). In Germany, direct or indirect contact of domestic pigs with wild boar is believed to have caused about 60 per cent of primary outbreaks (Moennig et al., 1999; Fritzemeier et al., 2000). However, scanty information is available regarding the endemics storm of CSF in wild pigs in Assam, India. Hunting of wild hogs is not routine for the rural people of this region. Thus collection of tissue samples from wild hogs is a rare opportunity for CSF screening. Present report covers three cases of CSF that were recorded in wild pigs in National sanctuaries in Assam as well as in adjoining state of India. The results are discussed from an epidemiological viewpoint.

Methods

Home land of Indian wild hog

Wild hog in India is well known as wild pig resembling the domestic pigs in the country region. Animals at maturity weight ranges from 220 to 450 lbs based on species. In Assam, 35.30% of the state’s geographical area comprising of 27 692 sq km is covered by dense forest. There are five national parks and 17 wildlife sanctuaries in which wild hogs are living. However, exact population sizes and densities of wild hog in these parks are not known. They are perceived as a pest for farmers and were considered vermin fit to be exterminated. Nevertheless, the blanket ban on hunting has lead to an increase in wild hog populations.

Case report

On three occasions CSF has been suspected in wild hog found dead in the forest. One outbreak was attended at Kaziranga National Park and the other at Manas National Park in Assam. The third outbreak was spotted at Jaldapara National Park in Assam–Bengal border district of West Bengal, India (Fig. 1). Five adult hogs were found dead in the forest, and three of the carcasses were putrefied. Pathological changes were recorded, and appropriate tissue samples were collected for CSF diagnosis (Fig. 2). As a part of seromonitoring of CSF virus antibodies at fringe areas, 18 serum samples were randomly collected from feral pigs adjacent to the National parks in Assam. Opportunistic sera samples were also collected from two captured wild hogs.

Classical swine fever diagnosis

Skeletons of wild animals as well as decaying remnants were found in the deep forests particularly during and after flood. During the month of February 2008 in Kaziranga national park, Assam as well as in Jaldapara, West Bengal, five dead carcasses of wild hogs were found by forest guards. In Manas national park, an ailing wild hog was captured and died subsequently. Post-mortem examinations of carcasses were carried out and recorded for any gross

Fig. 1. Distribution and location of National park and wildlife sanctuaries in Assam, India.
pathological changes. Representative tissue samples were collected from tonsil, lymph node, kidney, spleen, brain, lung, colon and distal part of ileum. Tissue samples were preserved in 10% formalin solution for histopathological examination.

Detection of CSFV antibody and antigen
For demonstration of CSFV antigen and antibodies, commercial CSFV antigen and antibody test kit (IDEXX, Bern-Liebeseld, Switzerland AG) were used.

Detection of CSF virus nucleic acid
Viral RNA was extracted from tissue samples using the QIAamp RNA Kit (QIagen, Hilden, Germany) following manufacturer’s instructions. Viral RNA was used for real-time PCR (RT-PCR) as shown earlier (Hoffmann et al., 2005). Samples with Ct values below 35 were considered positive. Two regions of the CSFV genome were used for phylogenetic analysis after partial sequencing: (i) a 150-nt fragment of the 5' UTR; and (ii) a 190-nt fragment of the E2 gene (Paton et al., 2000). Published primers were used to amplify these genes (Lowings et al., 1996) and Greiser-Wike et al. (1998)).

The amplicons of E2 gene and 5’ untranslated region (UTR) generated by nested PCR were subjected for purification using PCR purification kit (Qiagen) and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) for sequencing. Sequences were analysed by comparison with different CSF viruses available in GenBank using the BLAST tool from NCBI (Altschul et al., 1997). The multiple sequence alignment was carried out by CLUSTAL W algorithm (Thompson et al., 1994) and edited manually. Phylogenetic analysis was conducted using MEGA version 4.0.2 with bootstrap values corresponding for 1000 replicates (Tamura et al., 2007). Vaccine strain of India and other parts of world were extracted from GenBank to compare various reported CSFV sequences from field isolates. The phylogenetic tree was constructed by the neighbour-joining method using strains Cong Tremor and Kanagawa as outgroups. Representative samples from each outbreak were sequenced for E2 gene and 5’ UTR and submitted to GenBank (accession numbers: KF214683 and KF483865 for E2; KF551932 and KF551933 for UTR).

Results
Pathological findings
Grossly the affected animals observed were dehydrated. Ecchymotic haemorrhages were observed in the inner aspects of fore and hind limbs (Fig. 2a). The eyes found stuck with lacrimation and nostrils were smeared with discharge. Various lymph nodes and tonsils were swollen, oedematous and haemorrhagic. Pinpoint haemorrhages in the subcapsular region of the kidneys were found in almost all the cases. In spleen, raised haemorrhagic areas and typical infarct spots were observed (Fig. 2b).
pathognomonic button ulcers and haemorrhages were observed in the large intestine (Fig. 2c). The small intestine showed catarrhal enteritis. Mesenteric blood vessels were enlarged and congested. There was also the presence of petechial haemorrhage on the epicardial surface of the heart. The lesions in the brain were limited almost entirely to congestion and haemorrhagic in the meningeal surface. The presence of highly pneumonic lesions in the lungs was recorded (Fig. 2d). In most of the cases, congestion of the nictitating membrane was observed.

In the histopathological studies, kidney showed congestion of the blood vessels and focal areas of haemorrhages in the cortico-medullary regions. Kidneys showed interstitial nephritis characterized by the presence of infiltrating cells such as neutrophil, lymphocyte and macrophages in the interstitial spaces (Fig. 3a). There were focal degeneration and necrosis of the tubular epithelium. Moreover, focal areas of acute glomerular nephritis, characterized by swelling of the glomerular tuft, expanded up to the Bowman’s space with cellular infiltration was also observed.

In spleen, focal to diffuse haemorrhages were observed in the red pulp areas. In all five cases, follicular atrophy of the corpuscle, along with multiple focal or diffuse necrotic areas was found. The lymph nodes showed congestion of the blood vessel, along with focal to diffuse areas of haemorrhages. The Peyer’s patches in the ileum showed depletion of lymphocyte in the lymphoid follicles. Colon showed multiple ulcerative lesions (button ulcer) with necrosis of the epithelial mucosa. The lung showed lesions of interstitial pneumonia characterized by thickening of inter-alveolar septa with infiltration of mononuclear cells and congestion of alveolar capillaries.

Perivascular cuffing was observed in the cortical region of central nervous system characterized by mononuclear cells with congestion of the blood vessels (Fig. 3b). Meningitis, characterized by thickening of meninges with congestion and infiltrations by large number of mononuclear cells were present in all the cases.

Detection of CSFV antigen, antibody and nucleic acid

The nested PCR amplicons positive for E2 and UTR showed a product size of 271 bp (Fig. 4). Classical swine fever virus antigen and genome were detected by sandwich ELISA and real-time PCR in all five carcasses, respectively. The CSF antibody was detected by indirect ELISA (classical swine fever virus antibody test kit; Idexx) in fifteen of the eighteen feral pig sera while both wild hogs were found serologically positive for CSFV antibody, and the titres were ranged between 1 : 40 and 1 : 80.

Genetic analysis of the CSFV E2 gene revealed that the wild hog isolates KF483865 (Kaziranga) and KF214683 (Jaldapara) belonged to genotype 2.2 (Fig. 5). Other Indian isolates of CSF from domestic pigs of the north-eastern region (442 Kokrajhar, EU567073 and CSF Ar 3A) as well as from Uttar Pradesh (KC533779) also belonged to genotype 2.2. All these isolates clustered closely and share homology with strain 39 (AF407339) and ZJ-2/99 (FJ157197) from China and with isolates from Nepal (JX162241 and JX162240). Wild hog isolate Kaziranga and Jaldapara showed 97.9% identity with each other and nearly 96.8% with isolate JX162240 and JX162241 from Nepal collected in 2011. Wild hog isolate Kaziranga and Jaldapara showed nearly 96.3% identity with the Chinese Strain 39 (AF407339) and ZJ-2/99 (FJ157197).

Similarly, genetic analysis of the CSFV 5’UTR suggested that wild hog isolate Kaziranga (GenBank accession No KF551932) and Jaldapara (GenBank accession No KF551933) belonged to genotype 2.2 (Fig. 6). Four Indian isolates from domestic pigs in the North-east JF412663 (Manipur), JF412660 (Arunachal Pradesh), EU567080 (Mizoram) and JF412662 (Assam) detected during 2008–10, also belonged to genotype 2.2, along with strain EU567082, GU722592 and GU722590 from Bihar and EU567079 (Hisar), India. All these Indian isolates clustered with each other in the phylogenetic analysis (Fig. 6). Wild hog isolates Kaziranga and Jaldapara showed 100% identity with each other and 96.3% identity with Chinese Strain 39 (AF407339) and ZJ-2/99 (FJ157197).

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nucleotide sequence identity with each other and 99.3% nucleotide sequence identity with isolate from Northeast India, namely Manipur (JF412663), Arunachal Pradesh (JF412660), Mizoram (EU567080) and Assam (JF412662). Moreover, high nucleotide sequence identity was also observed with the strains from Bihar, a neighbouring state of Assam and West Bengal (EU567082, GU722591 and GU722590).

Discussion

The role of wild boar in maintenance and dissemination of CSFV infection to domestic pig population has been well established (Kaden et al., 1992, 2003). Furthermore, it has also been suggested that the infection is self-limiting in the wild hog metapopulation (Fritzemeier et al., 1998). However, in certain situations, the virus is circulating for years (Laddomada et al., 1994; Fritzemeier et al., 1998; Kern et al., 1999), particularly in an area where wild boar density is sufficiently high (EFSA, 2009). The outbreaks of CSF recorded in the National sanctuaries of Assam and adjoining wild sanctuary of West Bengal are the first report in this
part of India. Lack of information on abnormal mortality or about sick animals in wild hogs is due to the fact that wild hogs are not categorized under important wild fauna. Besides, farmers associated with agriculture as well as live-stock activities at fringe areas are unaware about seriousness of CSF. However, we have shown earlier the occurrence of CSF in domestic pigs and captive pygmy hogs from the entire north-eastern states of India (Barman et al., 2009, 2012). The present report is first comprehensive and the only document to link the ‘spill over’ and ‘reverse spill over’ of CSFV infection in wild and domestic pig population.

Dead carcasses of wild hogs were spotted by dedicated veterinarians and forest officials working in the jungle. Classical swine fever suspected wild hogs displayed comparable clinicopathology with that of CSF affected domestic pigs. As shown earlier, the outbreaks recorded here in three geographically different locations showed typical pathological CSF which were also observed in domestic pigs and pygmy hogs (Barman et al., 2012). As reported, CSF in wild hogs exhibits similar pathology as that of domestic pigs (Kern et al., 1999).

Monitoring and surveillance of wild hog metapopulations is an important clue for early suspicion and confirmation of CSF. Due to lack of awareness in the region chances of getting tissue samples from dead carcasses and blood from ailing hogs are scanty. The detection of the described cases was based on opportunistic samples. However, to depict clear epidemiology of CSF in wild, veterinarians, forest officials and farmers must truly be aware about the dynamics of CSFV infection.

Serological diagnosis of CSF is important for its detection in wild boar population, when acute clinical signs were absent. On the other hand, it is a tool for monitoring epidemiology of CSF in fringe areas. In the present study, evidence of CSFV antibody was demonstrated in wild and feral pigs. These findings confirmed the ‘spill over’ and ‘reverse spill over’ of CSFV infections among wild and domestic population in endemic areas. Nevertheless, more supportive active data need to be generated to establish the above noted phenomenon.

To the best of our knowledge, this is the first report of the association of CSFV genogroup 2.2 in wild population of North-east region of India. Past outbreaks recorded in different parts of India identified genogroup 1.1 in most of the cases (Barman et al., 2010; Patil et al., 2010; Sarma et al., 2011). The first evidence of genogroup 2 virus circulating in India was reported from samples collected in 2002–2004 (Desai et al., 2010). Thereafter, the prevalence of 2.2 genogroup was detected in 2006 from other states in the North, Central and Southern India (Barman et al., 2012; unpublished data). The shifting of CSFV genogroup from 1.1 to 2.2 in India was observed in this decade (2002–13). In Europe, shift CSFV from genogroup 1–2 has been reported in the 1980s (Paton et al., 2000). Similar findings have been reported from China (Tu et al., 2001) and Taiwan (Deng et al., 2005).

Interestingly, phylogenetic analysis of Indian isolates of domestic pigs and wild hog formed a single cluster both in E2 and 5′UTR gene sequences. All these isolates, including recent isolates of wild hog shared 97–100% identity suggesting circulation of genogroup 2.2 among domestic and wild pigs. In Europe, spread of CSFV from wild boar to domestic pig population was established (Teuffert et al., 1997; Ferrari et al., 1998; Fritzemeier et al., 1998). The most commonly adopting backyard pig farming in fringe areas of Assam and adjoining states creates a conducive environment for easy spreading of the virus. The present study insights the molecular epidemiology of CSFV of genogroup 2 circulation in wild boar populations.

It can be concluded that CSF is a recurring infection of domestic pigs and wild hog. Classical swine fever might be endemic in wild hog in Assam, India and might represent a permanent virus reservoir that poses a constant threat to domestic pigs. However, the specific role of wild hogs as CSFV reservoirs needs further investigation. Besides, backyard pig farming, highly mobile pigs and their products through live animal markets are main contributing factors in the endemic nature of CSFV in India. Presumably, any programme aimed for control and eradication of CSF at long term should include methodologies which allow not only an efficient approach in domestic animals but also in wild boar. Moreover, a non-invasive sampling strategy for monitoring wild hog population in Assam should be developed in view of the absence of hunting activities.

Conclusion

Classical swine fever is a recurring infection of domestic pigs and wild hog. Classical swine fever infection is endemic in wild hog in Assam, India and might act as a viral reservoir that poses a constant threat to domestic pigs. However, the specific role of wild hogs as CSFV reservoirs needs to be further clarified in our condition. Besides, backyard pig farming, highly mobile pigs and pig products through auction markets are the main contributory factors in epidemiology of CSF in India. Therefore, any programme aimed for control and eradication of CSF at long term should include methodologies which allow not only an efficient approach in domestic animals but also in wild boar. Due to the absence of hunting activities, a non-invasive sampling strategy for monitoring wild hog population in Assam should be developed.
Acknowledgement

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Molecular characterization of E2 glycoprotein of classical swine fever virus: adaptation and propagation in porcine kidney cells

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Abstract Classical swine fever virus (CSFV) is the causative agent of a highly contagious disease, hog cholera in pigs. The disease is endemic in many parts of the world, and vaccination is the only way to protect the animals from CSFV infection. The lapinized vaccine strains are occasionally not protective because of animal to animal passage, inadequate vaccination strategy, suboptimal vaccine dose, and emergence of new variants. The surface glycoprotein E2 of CSFV is a major antigenic determinant and can modulate the disease outcome in pigs. In the present study, we characterized the CSFV in porcine kidney cells. The CSFV vaccine strains showed enhanced replication following 15 passages in porcine kidney cells. Nucleotide sequence analysis of the E2 protein gene of the cell culture-adapted vaccine strain of CSFV showed a mutation in putative amino acid sequences that are identical to its virulent counterpart. The study suggests the possibility of exaltation in vaccine strains following its adaptation in host cells and paves the way for a further exploration of the biology of its outbreak.

Keywords Classical swine fever virus · E2 glycoprotein · Lapinized vaccine · Pig kidney cells

Report

Classical swine fever (CSF) is a highly contagious viral disease that mainly affects domestic pigs, wild boar, and pygmy hog (Dahle and Liess 1992; Artois et al. 2002; Barman et al. 2012). It causes huge economic losses to the pig industry worldwide. Although CSF has been eradicated from some parts of the world, a reservoir is maintained in wild boar populations and the virus can be reintroduced into domestic pigs, resulting in fresh outbreaks and the spread of the disease. The disease is endemic in India, and regular outbreaks have been reported in the northeastern part of the country which has adjoining borders with Bhutan, China, Myanmar, and Bangladesh (Barman et al. 2010). Animal migration from adjoining countries is very common and contributes to outbreaks in vaccinated herds. The mean prevalence of classical swine fever virus (CSFV) antibodies was 63.3% (376/594), whereas the viral antigen prevalence was 76.7% (220/287) from 594 samples collected from 12 states (Rahman 2011). Although the lapinized C strain vaccine is widely used, the high prevalence of CSF antibodies suggests that the disease is endemic in India and is a major threat to the pig industry. However, CSFV-specific antibodies in a flock could be due to vaccination. Moreover, vaccination outbreaks were also recorded on many occasions, raising a question on the efficacy of the
In the present study, we have analyzed the mutations in the E2 protein gene of live attenuated CSFV vaccines used in the field condition at the level of nucleotides as well as amino acid residues following its adaptation in porcine kidney cells. The study will help us in understanding the biology of the CSFV surface protein following its adaptation in porcine cells. Moreover, knowledge regarding mutations in the surface glycoprotein and its effect towards increased binding efficiency with the cell surface receptor can be a useful tool to design a better vaccine using a reverse genetics system.

The porcine kidney cells (PK-15) were procured from the ATCC (Manassas, VA). The cells were maintained in Eagle’s minimal essential medium (EMEM) containing 10% fetal calf serum (Invitrogen, Grand Island, NY) tested free for BVDV at 37°C in 5% CO₂. Three lapinized vaccine viruses, namely IVRI (Indian classical strain), Assam lapinized (Assam), and virus isolated from vaccinated pigs (local strain), were procured from the Department of Microbiology, College of Veterinary Science, Khanapara, Guwahati, India. The sequences of the three lapinized vaccine strains regularly used under field conditions were extracted from GenBank (accession numbers EU857642 (Indian classical strain), EU567078 (Assam strain), and KP195022 (local strain)).

The CSFV strains were passaged in PK-15 cells in 80% to 90% confluency blindly for 15 passages. For CSFV infection in PK-15 cells, 1 ml of virus inoculum treated with antibiotic and filtered through a 0.25-μm syringe filter was allowed to adsorb on the monolayer for 60 min. The EMEM containing 5% fetal calf serum tested free of pestivirus was overlaid after draining the virus inoculums from PK-15 cells. The cells are further incubated at 37°C under 5% CO₂ for 5 d. The infected PK-15 cells were freeze-thawed for three cycles and clarified at 3000 rpm, and the supernatant was collected as virus inoculum for the second passage and stored at −80°C.

The replication of CSFV strains in the PK-15 cells was analyzed by immunofluorescence (IF) and immunoperoxidase (IP) staining using the WH303 monoclonal antibody as described previously (Wensvoort et al. 1986; Barman et al. 2012). The CSFV titers in 50% endpoint tissue culture infectious dose (TCID₅₀) units/milliliter were determined on monolayers of PK-15 cells using the Reed and Muench method (Hierholzer and Killington 1996). The CSFV Indian classical strain, Assam strain, and local strain having a titer of 10³-25, 10⁴-200, and 10⁴-80 TCID₅₀/ml, respectively, were used for their first passage in PK-15 cells. Viral RNA was extracted from supernatants of infected cells by TRIzol (Invitrogen) using a standard protocol. Further, cDNA was prepared using a gene-specific reverse primer designed on the basis of the whole genome sequence of the CSFV strain: E2 rev (5'-CCG TTC GAG ACC AGC GGC GAG TTG-3') followed by PCR using E2 rev and E2 for (5'-CCC AAG CTT CGG CTA GCC TGC AAG G-3') primer (Mayer et al. 2003). The PCR-amplified products were sequenced directly using a BigDye

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Kumar et al. 2014: Vaccine in the study of the cell culture system.

Vaccine (Suradhat and Damrongwatanapokin 2003; Ji et al. 2014). Moreover, the efficacy of the vaccine depends on vaccination strategy, suboptimal vaccine dose, and hygienic measures (Elbers et al. 2001; Klinkenberg et al. 2003). In addition, animal to animal passage can modulate the disease outcome because of the mutation in the E2 gene that can alter the host immune response (Wehrle et al. 2007; Tamura et al. 2012; Fahnoe et al. 2014).

CSFV belongs to the genus Pestivirus under the family Flaviviridae (Thiel et al. 1993). The genome of CSFV is a positive-strand RNA of approximately 12.5 kb in length and is comprised of a single large open reading frame (Meyers et al. 1989; Becher et al. 1994). It is believed that the viral genes are translated as a large polyprotein that undergoes subsequent proteolytic processing by cellular and viral proteases (Rumenapf et al. 1991). Four structural genes, namely core protein (C), E⁰, E₁, and E₂, are found in the infectious virion. The C protein has RNA chaperone activity, and it binds to viral RNA in order to facilitate its packaging into the virion (Ivanyi-Nagy et al. 2008; Murray et al. 2008). The envelope of the CSFV contains three glycoproteins E⁰, E₁, and E₂ (Thiel et al. 1991). The E₂ protein in CSFV-infected animals is a major immunogen and contains conserved antigenic determinant regions (van Rijn et al. 1996; van Rijn 2007). The E₂ protein plays an important role in viral adsorption to host cells along with E⁰ and E₁ (Hulst and Moormann 1997; Wang et al. 2004). Virus neutralizing antibodies are raised against E₂ in CSFV-infected animals (de Smit et al. 2000; Qi et al. 2009). A cell line from a natural host is generally permissive for pestivirus replication in vitro, but considerable differences have been observed in its replication efficiency (Rohe and Edwards 1994; Flores et al. 1996). Moreover, mutations in the E₂ gene of vaccine strains are responsible for the adaptation of pestiviruses into a permissive cell line (Liag et al. 2003). The putative cell surface receptor for pestiviruses was identified as CD46, and its interaction with the envelope protein is important for endocytosis of the virion (Schelp et al. 2000; Maurer et al. 2004; Krey et al. 2005; Tews and Meyers 2007). The E₂ gene in CSFV encodes for a protein 373 amino acids long of 51 to 55 kDa size based on the level of glycosylation. The E₂ protein has four antigenic domains, namely A, B, C, and D, within the N terminus (Wensvoort 1989). The E₂ protein forms a homodimer and heterodimer with the E₁ protein during virus entry and attachment to the host cells (Zhang et al. 2006). The sequential epitopes of the E₂ protein elicit neutralizing antibodies, which direct protective immunity. Therefore, E₂ is explored as a candidate for DNA vaccines in order to prevent CSFV infection (Bouma et al. 1999; Qi et al. 2008; Qi et al. 2009). Variation in E₂ proteins can lead to the emergence of a new variant of CSFV (Chen et al. 2010; Leifer et al. 2012). No information is available regarding changes occurring in the lapinized CSFV strain following its adaptation in a cell culture system.
terminator v3.1 matrix standard kit and 3130xl Genetic Analyzer data collection software v3.0 (Applied Biosystems Inc). The E2 gene was sequenced at least three times from three independent RNA preparations to ensure a consensus sequence. Phylogenetic analysis of the E2 gene sequence of all the three strains with available GenBank sequences was performed by the Molecular Evolutionary Genetics Analysis software (MEGA4) using the maximum parsimony method. The robustness of the groupings in the neighbor-joining analysis was assessed with the 1000 bootstrap value (Kumar et al. 2008). The secondary structure of the putative E2 protein sequences was analyzed by PSIPRED software (http://bioinf.cs.ucl.ac.uk/psipred/).

The infection of CSFV vaccine strains did not show any cytopathic effect in PK-15 cells up to 15 passages. However, the CSFV strains showed positive IF and IP staining using the WH303 monoclonal antibody (Fig. 1). The CSFV Indian classical strain, Assam strain, and local strain showed a titer of $10^{7.25}$, $10^{5.00}$, and $10^{6.00}$ TCID$_{50}$/ml, respectively, after 15 passages. In addition, the replication of CSFV after each passage was confirmed by RT-PCR analysis using E2 gene-specific primers for the respective strains. The E2 gene sequences of all three vaccine strains following 15 passages were analyzed for their putative amino acid sequences. The nucleotide sequence of the E2 gene of the PK-15-adapted Indian classical strain showed 93.6% identity with its parental strain. Similarly, the nucleotide sequence of the E2 gene of the local strain and Assam strain showed 99.8% and 99.9% identity, respectively, with their parental strain after its adaptation in PK-15 cells (Table 1). The putative amino acid sequence of the E2 protein of the Indian classical strain and local strain of CSFV showed 95.6% and 99.4% identity, respectively, with the parental strain after its passage in PK-15 cells (Table 1). The CSFV strain Assam does not show any difference in amino acid level after its subsequent passage in PK-15 cells (Fig. 2). The local lapinized vaccine showed the mutation of N181K and W182L following its adaptation in PK-15 cells. A comparison of the amino acid sequences between the Indian classical strain and the reference sequence of the E2 protein of CSFV (NC_002657) showed differences of K720E, V744I, T886A, I971T, and A975E (Fig. 2). The conserved motif $^{829}$TA VSPTTLR$^{837}$ in domain A was found identical in all the strains independent of its number of passages in PK-15

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**Table 1** The nucleotide sequence and amino acid identity of the E2 protein gene of different strains of classical swine fever virus

<table>
<thead>
<tr>
<th>Protein</th>
<th>Indian classical</th>
<th>Indian classicalp</th>
<th>Local</th>
<th>Localp</th>
<th>Assam</th>
<th>Assamp</th>
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<td>Nucleic acid</td>
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<td>93.8 94.0 93.8</td>
<td>99.6</td>
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<tr>
<td>Amino acids</td>
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</table>

$p$ passaged virus
Figure 2  Amino acid sequence comparison of classical swine fever virus (CSFV) vaccine strains following its passage in porcine kidney cells (p indicates passaged virus). Amino acid sequence alignment was performed by ClustalW multiple alignment algorithm of the DNASTAR Lasergene 7 software package. GenBank accession number NC_002657 has been taken as reference sequence for CSFV. The E2 protein sequence encoded by the reference strain was listed as “E2 virulent” for the alignment in order to depict the correct amino acid position with respect to the CSFV polyprotein.
cells (Lin et al. 2000). Phylogenetic analysis of the E2 protein of cell culture-adapted vaccine strains with different CSFV strains showed clustering of the Indian classical strain with the local lapinized vaccine (data not shown). The amino acids $^{741}_7$, $^{763}_8$, $^{764}_9$, $^{804}_9$, $^{917}_9$, and $^{940}_9$ were found unique to the virulent strain and were absent from the vaccine strains (Fig. 2). Our result showed that these amino acids were unchanged even after 15 passages suggesting its specificity for the virulent CSFV strains. How these specific changes contribute to the altered phenotype observed for the CSFV E2 protein gene remains to be determined. However, it has been shown recently that two single amino acid changes, $^{763}_7$ and $^{968}_9$, in the E2 protein can cause the attenuation of CSFV in swine (Fahnoe et al. 2014). In addition, it has also been shown that T830A substitution in the E2 protein of CSFV can increase the pathogenicity (Tamura et al. 2012). Secondary structure analysis of the E2 protein of the PK-15-adapted Indian classical, local, and Assam strains showed the helix towards its carboxy terminus while the amino terminus is dominated by strands and coils similar to their non-passaged forms.

Our results of the cell culture adaptation of CSFV corroborate the earlier report where it has been shown that structural proteins can mutate faster than other viral proteins in order to selectively escape the host immune system (Wensvoort et al. 1990; Kwang et al. 1992). Moreover, the adaptation of CSFV in PK-15 showed subsequent high infectivity at a lower TCID$_{50}$ titer suggesting its better binding efficiency with the host cell surface receptor. A monoclonal antibody used in the study is targeted to the conserved amino acid sequences $^{820}_8$TAVSPPTTLR$^{837}_8$ and reacts equally with all three strains of CSFV. Our finding corroborates the earlier report where a change in amino acid sequences in the E2 protein contributed to the better binding efficiency of CSFV to the cell surface receptor (Liang et al. 2003). The conserved amino acid sequences $^{820}_8$TAVSPPTTLR$^{837}_8$ in all the CSFV strains as suggested earlier as linear epitope might not be important in the adaptation of the virus in vitro (Lin et al. 2000; Qi et al. 2008). The E2 protein is supposed to be a major determinant of virulence in CSFV, and changes in some critical amino acids might modulate its virulence (Risatti et al. 2005; Tamura et al. 2012). The existence of a conserved amino acid sequence in all three strains and their reactivity towards the WH303 monoclonal antibody suggest the possession of the immunogenic epitope in all three CSFV vaccine strains. However, the evaluation of cross-neutralization capacity in primary host sera with other vaccine strains and currently circulating wild strains can give the status of full protection in vaccinated animals. The study will be useful to understand the biology of CSFV and its repeated outbreaks in the vaccinated flocks. We have limited information about the biology of CSFV, and there are strains out there that can behave differently than what is taken as dogma. Further, exploring the mutations in the E2 protein gene of CSFV by reverse genetics will help us to modulate or attenuate the virus for the development of a better live recombinant vaccine.

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Application of nucleic acid based techniques for detection of
Classical swine fever virus: A comparative study

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Abstract: Classical swine fever (CSF) is a contagious and devastating viral disease, causing serious losses in the pig industry worldwide. In the control programmes of CSF, rapid detection and identification of the causative agent is a crucial step. Various PCR based techniques like nested RT-PCR, SYBR Green based Real-Time PCR and TaqMan based Real-time PCR were used for detection of CSFV nucleic acid in clinical as well as tissue samples. In our study three detection systems were tested for classical swine fever virus (CSFV) detection and for its discrimination from other pestiviruses; Nested PCR, non-specific dsDNA-binding SYBR Green dye based and specific fluorogenic TaqMan MGB probe based Real-Time PCR. However, one-step TaqMan Real-Time PCR assay was shown to be the most appropriate for pestivirus discrimination in comparison to the other two assays, moreover, it reduces the risk of contamination and is less time consuming.

Keywords: Classical swine fever virus, Nested PCR, Pestivirus, Real-time PCR, SYBR Green, TaqMan probe

I. Introduction

Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), a disease notifiable to the Office International des Epizooties (OIE) according to the Terrestrial Animal Health Code (www.oie.int). CSFV is a member of the genus Pestivirus in the family Flaviviridae. The genus also includes Bovine viral diarrhoea virus 1 (BVDV-1), Bovine viral diarrhoea virus 2 (BVDV-2), Border disease virus (BDV), and a tentative species, Pestivirus of giraffe [1]. CSFV is an enveloped virus with a single stranded, positive-sense RNA genome of approximately 12.5 kb in size. The genome contains two conserved untranslated regions (UTR) at the 5' and 3' ends, and a single open reading frame (ORF) encoding a polyprotein of about 4000 amino acid residues. The polyprotein is processed into 12 polypeptides by viral and cellular proteases [2, 3]. The envelope of the CSFV contains three glycoproteins Erns, E1, and E2 [4]. The E2 protein in CSFV-infected animals is a major immunogen and contains conserved antigenic determinant regions.

CSF is a highly contagious disease that is mainly spread by contacts between pigs or by feeding pigs with contaminated meat. It is very important to differentiate between CSFV and BVDV or BDV infections in pig herds. This is why rapid, sensitive and specific laboratory diagnostic methods are needed to confirm outbreaks of CSF.

II. Materials And Methods

2.1. Collection of samples: Different outbreaks that occurred in Assam and adjoining states during 2013-14 were attended and whole blood were collected from clinically affected pigs in EDTA containing vacutainer. A total of 65 blood samples were processed with three different detection assays for CSFV.

2.2. Detection of CSFV nucleic acid in clinical samples

2.2.1. RNA extraction: Viral RNA was extracted using the QIAamp Viral RNA Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions.

2.2.2. Reverse transcription (RT): cDNA synthesis was done by using High Capacity cDNA Reverse Transcription Kit (Invitrogen) as per manufacturer’s instructions. The cDNA was further used for E2 Nested RT-PCR and SYBR Green based Real-Time PCR.
2.2.3. Nested RT-PCR: For Nested RT-PCR four sets of primers described earlier [5] were used targeting E2 gene of CSFV. The primary and nested PCR reaction was carried out in standard 50 µl reaction mixture as per the method described in European Union Diagnostic Manual. For confirmation of Nested RT-PCR amplicons gel electrophoresis was carried out in 1.7% agarose gel containing ethidium bromide in 0.5X Tris borate EDTA and visualized on a UV transilluminator as per standard procedures. For size comparison, a 100bp DNA ladder marker was run parallel to the PCR amplicons.

2.2.4. SYBR Green based Real-Time PCR: SYBR Green based Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) as per manufacturer’s instructions in a 7300 Real Time PCR system (Applied Biosystems) with Pan pesti specific primer sets (Table 1).

2.2.5. TaqMan MGB probe based Real-Time RT-PCR: The TaqMan Real-Time assay was carried out using SuperScript III Platinum One-step Quantitative RT-PCR kit (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions in a 7300 Real Time PCR system (Applied Biosystems). A set of primers and a probe described earlier [6] targeting the highly conserved CSF sequence was selected for the Single Step TaqMan Real-Time RT-PCR assay.

Positive and negative extraction and amplification controls were included in each evaluation.

III. Result And Discussion

Nested RT-PCR: Out of total 65 clinical samples screened 39 samples were detected positive in E2 Nested PCR. The Nested PCR amplicons of E2 shows a product size of 271 bp (Fig. 1). The detection time was calculated to be approximately 6 hour and 30 minutes.

Real-Time PCR using SYBR Green method: 50 out of 65 samples were found positive by SYBR Green Real-Time PCR. Samples showing threshold cycle (Ct) values below 35 were designated positive, and samples with Ct values above 35 were considered negative. SYBR Green Real-Time assay can be non-specific and less accurate as intercalating dyes (SYBR Green I dye) generate fluorescence when bound to any dsDNA products including primer dimers. Therefore, to avoid false positive signals non specific product formation were again checked using dissociation curve or gel analysis. Amplification from specific product is displayed with a Tm of 84 °C, while primer dimer product has a characteristically lower Tm of 75 °C (Fig. 2). For SYBR Green Real-Time PCR detection time was calculated to be approximately 4 hours.

Real-time RT-PCR using TaqMan MGB probe: 45 out of 65 samples were found positive by TaqMan Real-Time assay. The samples showing threshold cycle (Ct) values below 35 were designated positive, and samples with Ct values above 35 were considered negative (Fig. 3). Since single step TaqMan assay is sequence-specific probe-based detection system where the probe is designed to bind within the amplified PCR fragment, competing side reactions such as primer dimerization and mispriming were significantly avoided and the no template controls and negative samples did not result in an increase in fluorescence above the baseline. For single step TaqMan Real-Time Assay detection time was approximately 2 hour and 20 minutes.

IV. Figures And Tables

![Fig. 1. Gel electrophoresis of E2 PCR amplicons](image)

Lane1: Negative control; Lane2: Positive control; Lane 3: 100 bp marker; Lane 4: Positive sample
Application of nucleic acid based techniques for detection of Classical swine fever virus.

Table 1. Pan pesti specific primer sets used in SYBR Green based Real-time PCR

<table>
<thead>
<tr>
<th>Primer Identity</th>
<th>Sequence (5'-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Primer</td>
<td>TGG GTG GTC TAA GTC CTG AGT</td>
<td>G. Saikumar</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>GTG TGA TTT CAC CCT AGC GA</td>
<td></td>
</tr>
</tbody>
</table>

V. Conclusion

In this study, the performances of a gel-based nested RT-PCR assay and two Real-Time based assays were evaluated for detection of CSFV.

It has been shown that molecular based technique using E2 specific primer could specifically detect CSFV genome in higher percentage comparing with that of the polyclonal antibody based techniques like direct FAT and S-ELISA [7]. In addition, it has been shown that the order of sensitivity for different tests in detection of CSFV varies as RT-nested PCR>RT-PCR>Virus isolation>ELISA [8]. The gel-based RT-PCR assay is the method of choice for rapid detection of CSFV especially for laboratories that cannot afford a Real-Time machine.

Pan pesti specific SYBR Green Real-Time PCR detected the highest number of positive samples followed by TaqMan Probe based Real-Time PCR and E2 Nested PCR. Samples with very low viral load detected negative in E2 Nested PCR were detected positive by both the Real time assays because of their higher sensitivity. Comparing the two Real-Time PCR detection systems, both non-specific dsDNA-binding dye SYBR Green and specific fluorogenic TaqMan MGB probes reduced the risk of contamination and was less time consuming. However, SYBR Green based Real-Time PCR being pan pesti specific could detect samples other than CSFV i.e. BVDV or BDV. ButTaqMan Probe based Real-Time PCR being sequence-specific probe-based detection system detected only CSFV specific positive samples. Therefore, Real-Time RT-PCR with TaqMan MGB probes is more suitable for CSFV detection and for its discrimination from other pestiviruses than the Real-Time assay with SYBR Green.

Unlike a nested, or two-step SYBR Green RT-PCR assay, Single step TaqMan assay is much simpler, more convenient and easier to undertake. The combination of the reverse transcription (RT) and PCR steps into one step greatly reduces time-consuming procedures and eliminates additional manipulations that are normally required for a two-step reaction. The risk of carryover contamination can be minimized since reaction tubes are not required to open for RT step or at the end of the run for gel electrophoresis.

In summary, the one-step TaqMan Probe based Real-Time PCR assay provides a simple, fast, highly sensitive and specific method for detection of CSFV in clinical samples or as confirmatory tests for other assays, such as virus isolation, or antigen ELISA. Moreover this diagnostic tool, using EDTA or Heparin treated blood from live animals, allowed for rapid identification of CSF infected pigs and could save a lot of time when control measures have to be applied to prevent virus dissemination between herds, after the first detection of the virus.
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Comparative evaluation of single step real-time RT-PCR and gel based RT-PCR assay for detection of classical swine fever

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ABSTRACT

To control classical swine fever (CSF), a highly contagious viral disease causing serious losses in the pig industry worldwide, rapid detection and identification of the causative agent is a crucial step. In the present study, real-time RT-PCR and gel based RT-PCR techniques were compared for detection of CSF virus nucleic acid in both clinical as well as tissue samples. Clinical and tissue samples (325) were collected from CSF suspected outbreaks from different part of north eastern region of India. In gel based RT-PCR, % positivity was 44.61% while in real-time RT-PCR it was 57.23%. Highest per cent positivity was recorded in tonsil followed by mesenteric lymph node, blood, nasal swab, spleen, kidney and ileum. The study indicated that probe based RT-PCR could specifically detect CSF virus genome and detection limit was about one log higher than a gel based PCR assay targeting the non-translated region. Total time required to complete the gel based RT-PCR including extraction of viral RNA was about 6 h. On the other hand, real-time RT-PCR assay can be performed in 2 to 3 h, thus providing a rapid detection tool.

Key words: Classical swine fever, CSFV, Gel based RT-PCR, Real-time RT-PCR

Classical swine fever (CSF), a highly contagious and enzootic disease in most of the pig producing states, particularly in the north eastern states of India, is caused by the classical swine fever virus (CSFV), under the genus Pestivirus. Pestivirus consists of one large open reading frame flanked by highly conserved 52 and 32 non-translated regions (NTR) and the CSFV, BVDV and BDV can be differentiated by the sequences of their 52 NTR (Hoffmann et al. 1994).

The clinical picture of CSF infection is highly variable and rapid and highly sensitive laboratory methods are essential for detection of CSF. Detection of viral nucleic acid by polymerase chain reaction (PCR) meets the criteria of speed and high sensitivity and a gel based nested reverse transcription-PCR (nRT-PCR) assay for the diagnosis of CSF was found sensitive and specific, but its performance was not compared to other molecular assays. While with fluorogenic probe, detection of sequence specific templates can be achieved in real-time, specificity is ensured by an inherent hybridization reaction and cross contaminations are largely avoided (Heid et al. 1996). Therefore in the present study, a gel based nested RT-PCR and real-time RT-PCR targeting the 52 NTR of CSFV genome were compared in detecting CSF virus in both clinical and tissue samples.

MATERIALS AND METHODS

Collection of samples: Clinical samples like anticoagulant added blood and nasal swab and postmortem tissue samples like tonsil, mesenteric lymph node, spleen, kidney and ileum from dead pigs suspected for CSF infection were collected from different part of north eastern regions without using any preservative and stored at –80°C till further processing. Tissue samples collected from dead pigs were processed in the laboratory as per European Union Diagnostic Manual (Anon 2007).

RNA extraction: Classical swine fever viral RNA was extracted from clinical and postmortem tissue samples, cell culture propagated CSF virus, positive and negative controls using viral RNA kit according to the manufacturer’s instructions. Quantity of fluid sample (140 ml) was used for RNA extraction. Finally, the RNA pellet was eluted using 60 ml elution buffer and stored at –20°C till further use.

Reverse transcription: The 6 ml of RNA was subjected to cDNA synthesis using random primer (50 ng/ml) and nuclelease free water to make 13 ml reaction volume and subjected to 70°C for 5 min and later hold on 4°C and briefly spun. Thereafter, 4 ml of 5 × RT buffer, 1 ml of RNase
inhibitor (40 U/ml), 1 ml of 10 mM dNTP mix and 1 ml of reverse transcriptase (200 U/ml) were added and incubated at 25°C for 10 min followed by 42°C for 1 h. The reaction was stopped by inactivating the reaction mixture at 70°C for 10 min. RNA and cDNA concentration was measured using nano drop spectrophotometer in ng/l and quality was checked as a ratio of OD 260/280.

**Gel based nested RT-PCR assay:** The primers used for primary and nested RT-PCR targeting 5′ NTR were designed from the genome of Alfort-187 strain of CSFV (Greiser-Wilke et al. 1998). The primary PCR was performed in 50 ml reaction volume containing 10X Taq buffer, 25 mM MgCl₂, 10 mM dNTPs mix, 5 ml of cDNA, 20 pmol of each Primer I (5′ CTAGCCATGCCCCWYA GTAGG 32, nucleotide position 94–113) and Primer II (5′ CAGCTTCARYGTTGATTGT 32, nucleotide position 514–496) and 2.5 U of Taq DNA polymerase and nuclease free water. The reaction was carried out for 34 cycles in a thermal cycler. Each PCR cycle consisted of 30 sec of denaturation at 95°C, 45 sec annealing at 58°C and 1 min extension at 72°C. The CSFV virus strain RNA and RNA extracted from tonsil of caesarean derived old piglet was used as positive and negative control respectively.

For nested PCR, 5 ml of primary PCR product was amplified using Primer III (5′ AGCTTCTCCTTGGTGG TCTA 32, nucleotide position 146–163) and Primer IV (5′ TGGTTGCTTA GCTGTTGTA T 32, nucleotide position 141–166) and subjected to thermo cycling conditions as primary PCR except annealing at 56°C. The nested PCR products along with positive control and negative control were subjected to electrophoresis in 1.7 % agarose gel containing ethidium bromide at 78 V for 1 h in 0.5 X Tris borate EDTA buffer. The DNA bands were visualized on a UV transilluminator. For size comparison, a 100 bp DNA ladder was run parallel to the PCR amplons.

**Single step TaqMan real-time RT-PCR assay:** Published oligonucleotide primers and the fluorogenic probe to target a highly conserved region within the 52 NTR of the CSFV genome were used in this study. The locations and sequences of the primers and probe according to CSFV Alfort-187 genome were used in this study. The specificity was particularly enhanced because this technique almost and always eliminates any spurious non-specific amplification product. Thus the desired target sequence alone was amplified.

Again, no amplification in the primary or nested PCR in negative control tissue indicated specificity of PCR product obtained in clinical and postmortem samples. Barman et al. (2012) used same primers for confirmation of CSF virus in the pygmy hog.

In nested RT-PCR 325, clinical and postmortem tissue samples were processed for amplification of 52 NTR fragment of CSFV. About 44.61 % samples were found positive for CSFV and highest percent of samples positivity was demonstrated in tonsil (60.00%) followed by mesenteric lymph node, blood, spleen, nasal swab, kidney and ileum (Table 1). Van Oirschot (1992) stated that in the first viraemic phase, almost all the lymphoid organs including tonsil, mesenteric lymph node and spleen were infected and propagated to highest concentration. Present study also justified frequent demonstration of virus in the lymphoid organs. The CSF virus is in general detectable in blood and organ samples for weeks after infection.

Table 1. Detection of classical swine fever virus in clinical and tissue samples by Gel based RT-PCR and real-time RT-PCR

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>No. of sample tested</th>
<th>No. of sample positive in Gel based RT-PCR (%)</th>
<th>Real-time RT-PCR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>66</td>
<td>30 (45.45)</td>
<td>39 (59.09)</td>
</tr>
<tr>
<td>Nasal swab</td>
<td>28</td>
<td>12 (42.85)</td>
<td>16 (57.14)</td>
</tr>
<tr>
<td>Tonsil</td>
<td>25</td>
<td>15 (60.00)</td>
<td>18 (72.00)</td>
</tr>
<tr>
<td>Spleen</td>
<td>86</td>
<td>38 (44.18)</td>
<td>48 (55.81)</td>
</tr>
<tr>
<td>Mesenteric lymph node</td>
<td>28</td>
<td>16 (57.14)</td>
<td>18 (64.28)</td>
</tr>
<tr>
<td>Kidney</td>
<td>81</td>
<td>30 (37.03)</td>
<td>42 (51.85)</td>
</tr>
<tr>
<td>Ileum</td>
<td>11</td>
<td>4 (36.36)</td>
<td>5 (45.45)</td>
</tr>
<tr>
<td>Total</td>
<td>325</td>
<td>145 (44.61)</td>
<td>186 (57.23)</td>
</tr>
</tbody>
</table>
Irrespective of the strain specific virulence, CSF wild type virus was routinely amplified in blood samples for more than 3 weeks by Handel et al. (2004), who recommended RT-PCR assay for the detection of pigs infected with low virulent field virus strains.

The TaqMan based real-time RT PCR for the clinical and tissue sample was carried out using the set of primers and probe designed by Hoffmann et al. (2005). To minimize the risk of contamination, one step RT-PCR protocol was carried out and the cut off C<sub>T</sub> value was taken after 50 cycles. The complete run takes approximately 2 h. The real-time PCR analysis showed that out of the total 325 samples, detection of CSFV was possible in 57.23 % samples (Table 1). Out of the 57.23 % positive samples, highest percent positivity was recorded in tonsil (72.00%) followed by mesenteric lymph node, blood, nasal swab, spleen, kidney and ileum. No false negative result was obtained with real-time RT-PCR since all the samples assigned negative by real-time RT-PCR were also negative by nested RT-PCR. On statistical analysis significant difference was observed between both the tests (P<0.05). Ten-fold dilution series of cell culture adapted CSF vaccine virus containing 5.5 TCID<sub>50</sub> were tested in parallel in the real-time RT-PCR as well as in nested RT-PCR assay. An amplified product of 93 bp and 271 bp fragments located in the 52 NTR region of CSFV genome was generated in real-time RT-PCR and in nested RT-PCR respectively. The detection limits of both the assays were compared. In terms of sensitivity a one log unit (10 times) was increased in real-time RT-PCR as compared to the nested RT-PCR. The amplification plot of test sample in real-time PCR is shown in Fig 1.

Thus PCR methods offer a promising alternative in the diagnosis of CSF and differentiation of the virus from other Pestiviruses (Hoffmann et al. 2005). Due to the high proportion of detected animals, nested RT-PCR is especially suitable for early diagnosis on individual pigs, which can be very useful for the animal trade.

But major drawback of the nested RT-PCR is that the risk of carryover contamination and time consumed as it takes 6 to 7 h to complete the assay. However, rapid detection of infected pig holdings is of paramount importance in stopping the further spread of CSF. To overcome this problem real-time RT-PCR is used, which is an accurate, rapid and reliable method for the detection of CSFV. It also eliminates post-PCR processing of PCR products. This helps to increase the throughput, reduces the chance of carryover contamination and disables post-PCR processing. In the present study, real-time RT-PCR alone could additionally detect about 12.62 % more positive samples than that of nested RT-PCR. The real-time PCR can be carried out even with a poor quality sample in which the pH has altered or even putrefaction has initiated as such sample may still have sufficient intact RNA. The real time RT-PCR protocol used was rapid and specific in detection of CSFV genome where results could be obtained within 2 hours. Jamnikar Ciglenecki et al. (2008) also reported that TaqMan real time PCR is more sensitive for virus detection in clinical samples from naturally infected animals. This is an important feature when definitive diagnostic results are required in a short timescale during emergencies.

Based on the results, it was concluded that the real-time RT-PCR assay provides a rapid, highly sensitive and specific method for detection of CSFV in both clinical and tissue samples. The early detection of CSF by real-time RT-PCR suggested potential use in disease control, as screening assay for monitoring a disease outbreak in real time. However, the real-time RT-PCR assays enable handling with several samples at the same time and provide results with greater sensitivity in comparison to gel based RT-PCR.

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Short communication

Development of single dilution immunoassay to detect E2 protein specific classical swine fever virus antibody

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Affinity purification
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A B S T R A C T

Classical swine fever virus (CSFV) is the causative agent of a highly contagious disease in swine. The disease is endemic in different parts of the world and vaccination is the only way to protect pigs from CSFV infection. The virus surface protein E2 is the major immunogenic protein eliciting protective immunity against CSFV infection in swine. The whole virus antigen cannot differentiate CSFV from other pestiviruses as it cross reacts with border disease and bovine viral diarrhea virus. Commercial available ELISA is based on the whole CSFV particle and can lead to false positive results. Moreover, the available commercial ELISA is not cost effective. In the present study, a recombinant E2 protein based single serum dilution ELISA was developed which showed enhanced sensitivity, specificity and accuracy as compared to commercial CSFV detection ELISA. The recombinant E2 protein based ELISA could be an alternate to existing diagnostics against CSFV infection in pigs.

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Classical swine fever virus (CSFV) is the causative agent of a highly infectious disease in swine. The classical swine fever (CSF) or hog cholera can cause huge economic impact to the pig industry worldwide (Moennig, 2000). It affects both domestic and wild pigs with varying degrees of pathogenicity. Although CSF is distributed throughout the world, it has mainly been reported from Asia, parts of Africa, Central and South America and Europe (Artois et al., 2002; Barman et al., 2014; Flores-Gutierrez and Infante, 2008). Frequent outbreaks of CSF have been reported from different parts of the world, including Belgium (1990–94), Germany (1993–2000), Italy (1995–97) and the Netherlands (1997). Recently, CSF outbreaks have been reported from Madagascar, Singapore, Laos, Lithuania, Myanmar, Colombia, and Republic of Korea (Ji et al., 2015). However, the disease has been eradicated from Australia, North America, and New Zealand (Anonymous, 2008).

CSFV belongs to the family Flaviviridae under genus Pestivirus. CSFV is an enveloped virus having a positive strand RNA with an approximately size of 12.5 kb and comprises of a single large open reading frame (ORF) (Meyers and Thiel, 1996). The genome of CSFV is flanked by two untranslated regions (UTRs) flanking the entire ORF which encodes a polyprotein of approximately 3900 amino acids (Meyers et al., 1996). This polyprotein gives four structural (C, Erns, E1, E2) and eight nonstructural proteins after processing by the cellular and viral proteases (Npro, P70, NS2, NS3, NS4A, NS4B, NS5A, NS5B) (Lowings et al., 1996; Meyers and Thiel, 1996). Npro protein is a non-structural protein, which functions as a cysteine protease (Bauhofer et al., 2005). Erns is a glycoprotein secreted from the cells infected by CSFV and is known to inhibit the interferon induction by the host cell (Matzener et al., 2009; Weiland et al., 1999). E1 is a type I trans-membrane protein involved in the adsorption of virus to the host cells (Fernandez-Sainz et al., 2009). CSFV encodes another small hydrophobic protein P7, which is flanked by sequences that are recognized by signal peptidase and is essential for the production of infectious virus (Moser et al., 1999).

E2 is an envelope glycoprotein present on the surface of CSFV and is important to induce host immune response during infection (Qi et al., 2009; Zhang et al., 2006). The E2 protein contains conserved antigenic determinant regions and it is the major immunogenic protein eliciting protective immunity against CSFV infection in swine (Greiser-Wilke et al., 1990; Rumenapf et al., 1991; van Rijn et al., 1996). E2 protein contains two linear B cell epitopes YYEP and TAVSPTTLR spanning towards its carboxy and amino terminus, respectively. The amino acid sequence motif YYEP is specific to pestivirus while TAVSPTTLR is specific to CSFV (Lin et al., 2000; Yu et al., 1996). In addition, E2 protein is accompanied with four relatively independent antigenic domains (A, B, C and D). The domain A has three subdomains (A1, A2 and A3). The E2

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is frequently used to design DNA vaccines against CSFV by different research groups (Beer et al., 2007; Bouma et al., 1999; Qi et al., 2008). Structurally, the E2 protein forms a homodimer during entry and heterodimer with E1 while attachment of the viral particles to the cell (Zhang et al., 2006).

In epidemiological surveys, detection of virus specific antibodies in serum samples is important in order to monitor the circulation of wild CSFV in population. Neutralizing assay is the test of choice to detect CSFV infection in the laboratory. However, detection of neutralizing antibody is time consuming, needs skill and well set up cell culture laboratory. Development of single dilution indirect ELISA can be a convenient alternative tool to detect CSFV specific antibody in pig sera (Li et al., 2013; Yang et al., 2012) using complete E2 protein as detecting antigen. Single serum dilution could be better than serial dilution method because it requires less time, chemical and plasticware making it a cost effective assay. Here we are reporting the expression of complete E2 protein using the bacterial expression system. The bacterial expressed E2 protein showed efficient binding with monoclonal antibodies and with the serum collected from the field outbreaks. The study will be useful in designing an efficient diagnostics against CSFV infection.

The porcine kidney cells (PK-15) were procured from ATCC (Manassas, USA). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum and essential antibiotics at 37°C in 5% CO2. The CSFV vaccine strains were procured from college of veterinary science, Guwahati, India. The virus was grown in PK-15 cells using standard protocols (Hulst et al., 2000). The infectious virus particles were recovered by repeated freeze and thaw following by filtering the extracted supernatant through 0.22 μm membrane filter. The stock of the virus was stored at −80°C for subsequent use. Titration of the virus was done in 96-wells microtitre plate containing PK-15 cells. Virus vaccine was diluted ten-fold in DMEM containing 10% calf serum into each of the five wells in a 6-well plate. The titer of virus stock was calculated by immunoperoxidase assay using CSFV monoclonal antibody after 72 h post-infection as described earlier (Mittelholzer et al., 1998).

Viral RNA was extracted using Trizol (Invitrogen, USA) according to the standard protocol. The cDNA was synthesized from extracted RNA using superscript II RT (Invitrogen, USA) and gene specific reverse primer (5′ CGCGTCTGATCAACCGCGGATGTTCG 3′) designed from available GenBank sequence (Accession number NC_002657). The E2 gene sequence was PCR amplified using E2 forward (5′ CGCGTCTGATCAACCGCGGATGTTCG) and E2 reverse (5′ CGCGTCTGATCAACCGCGGATGTTCG 3′) primers. Underline sequences are complementary to the CSFV genome and italics sequences are the restriction site EcoRI and XhoI, respectively. The amplified PCR products were purified by QiAquick gel extraction kit (QIAGEN, Germany) and sequenced by BigDye terminator v.3.1 matrix standard kit and 3130xl Genetic Analyzer Data Collection software v3.0 (Applied Biosystems, USA). The data extracted from analyzer was analyzed by DNAsStar software (DNASTAR Inc, USA).

The complete E2 protein gene was cloned into pET28a prokaryotic expression vector (Novagen, Germany) flanking EcoRI and XhoI restriction sites. The pET28a has His tagged at the N terminal which was used to purify the E2 protein by affinity chromatography. The integrity of the E2 gene after cloning was confirmed by sequencing. The pET28a containing complete E2 gene was transformed into Escherichia coli BL21 (DE3) pLYsS cells (Novagen, Germany) and induced by 1 mM isopropyl-β-D-thiogalactoside (MBI Fermentas, Germany). The fusion protein was extracted after induction of transformed BL21 cells for 4 h at 37°C and purified by affinity chromatography containing Ni-NTA (Invitrogen, USA). The recombinant protein was further purified using the Amicon ultra-4 centrifuge filter device (Millipore, USA) with a membrane cut-off of 10 kDa and further dialyzed using 10% glycerol in PBS. The concentration of the purified protein was determined by modifying Lowry’s Protein assay kit according to the manufacturer’s protocol (Pierce, USA). The expression of the recombinant E2 protein was further confirmed by SDS-PAGE and western blot using an anti E2 monoclonal antibody WH 303 (Wensvoort et al., 1986).

Two rabbits were immunized subcutaneously with 0.5 mg of purified recombinant E2 protein emulsified in Freund’s complete adjuvant (Sigma, USA) for the preparation of polyclonal antibodies. Subsequent booster was given by purified recombinant E2 protein with Freund’s incomplete adjuvant (Sigma, USA) for 2 occasions at 14 days interval. The sera sample was collected 14 days after final injection and stored at −80°C. The antibody against recombinant E2 protein was confirmed by western blot using goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce, USA) as well as by commercial CSF kit (HerdChek, IDEXX, USA). A total of 210 pig serum samples were screened for the CSF infection. Blood samples of suspected pigs were collected under aseptic condition and serum were separated after centrifugation at 1500 × g for 20 min, and the sera were stored at −20°C. The sera was analyzed for swine flu and porcine reproductive and respiratory syndrome virus using a commercial kit (PRRS X3 and influenza A kit from IDEXX, USA).

96-well flat bottom polystyrene plates (Greiner, USA) were coated with recombinant E2 protein (3 μg/well) using nitrate buffer (pH = 9.6) and incubated at 4°C overnight. Plates were washed with phosphate buffer saline containing Tween-20 (PBST) and blocked with 5% lacalbumin hydrolysat for 1 h at 37°C. Optimal concentration of E2 protein was determined by the checkerboard titration method (Robinson et al., 1985). Pig serum samples were initially diluted 10 times and then diluted serially and incubated at 37°C for 1 h. The plates were then washed with PBST, and incubated with 100 μl of the HRP-conjugated anti-pig antibody raised in rabbit (Pierce, USA) for another 1 h at 37°C temperature. The plates were washed and the E2 protein binding with serum samples was detected with 100 μl of TMB (Invitrogen, USA) for 15 min at 37°C temperature. The enzymatic reaction was stopped by 100 μl of 2 M H2SO4, and plates were read at 450 nm in a microtitre plate reader (Biotek, USA). Any serum sample showing an OD above the mean +3 standard deviation of the negative wells was considered positive. The negative serum samples were used to construct positive-negative threshold (PNT) baseline as shown earlier (Snyder et al., 1983). Similarly the positive curve was also plotted. The absorbance of the test sample dilutions was calculated using the formula:

\[ S_{\text{P ratio}} = \frac{OD_{\text{sample}} - OD_{\text{negative}}}{(OD_{\text{positive}} - OD_{\text{negative}})} \]

The PNT line was calculated using negative serum samples which were screened negative by commercial kit. The collected negative serum samples were diluted and resultant OD values were plotted against dilution. The resultant PNT line was used to find out the titer for the known positive serum samples by the sub- traction method as described earlier (Snyder et al., 1983). The OD values obtained for every logarithmic dilution was compared with observed titer and the highest correlation coefficient was selected to calculate the titer from that dilution. The constants like slope and intercept were calculated by the scatter plot as described earlier (Snyder et al., 1983).

The sensitivity, specificity and accuracy of the single dilution sera in comparison to the commercial CSFV diagnostic are determined using following formulae.

Sensitivity = \( \frac{x}{x + y} \times 100 \)

where, ‘x’ is the number of sera positive by commercial CSFV diagnostic ELISA and single dilution ELISA; ‘y’ is the number of sera
negative by our test ELISA and positive by commercial CSFV diagnostic ELISA.

Specificity = \( \frac{\alpha}{\alpha + \beta} \times 100 \)

where, \( \alpha \) is the number of sera negative by commercial diagnostic ELISA and single dilution ELISA; \( \beta \) is the number of sera positive by single dilution ELISA and negative by commercial diagnostic ELISA.

Accuracy = \( \frac{x + \alpha}{x + y + \alpha + \beta} \times 100 \)

The PCR amplified product of CSFV E2 protein was successfully cloned into pET28a vector and showed release of desired fragment upon digestion with EcoRI and Xhol. Furthermore, the sequencing of the E2 protein showed intactness of the nucleotide sequence. The recombinant E2 protein expressed in BL21 cells as a fusion protein with His tag was about 56 kDa in size (Fig. 1A and B). The average yield of the recombinant E2 protein was 70 mg/l of the bacterial culture used for its purification. The recombinant E2 protein showed the corresponding band of 56 kDa in western blot using raised polyclonal rabbit sera (Fig. 1C). The ELISA results and indirect immunoperoxidase assay confirmed the specificity for raised polyclonal antibodies (data not shown).

The calculated OD values were plotted on Y-axis against different serum dilutions in X-axis. The point where the sample line cuts the PNT line is taken as titer of the sample. The PNT baseline with different positive serum samples is presented diagrammatically and single serum dilution was calculated by linear regression (Fig. 2). The checker board titration of purified recombinant E2 protein showed 50 ng/well of protein being optimized for working range. The PNT curve for the calculation of the sample serum titer was obtained manually. ELISA using the single serum dilution method was performed using 50 serum samples. The correlation coefficient of 0.9349 was calculated at 1:80 dilutions that were more than the calculated value for all other dilutions. Thus, 1:80 dilution of serum samples was further considered to predict the titre of remaining serum samples. The slope and intercept were 3.8246 and 0.0958, respectively. The observed and predicted OD for all the serum samples were calculated using the regression equation, Observed OD = 3.8246(predicted OD) + 0.0958. A linear relation was observed between the predicted and observed titres (Fig. 3).

![Fig. 1.](image1.png) Fig. 1. Expression analysis of recombinant E2 protein of classical swine fever virus (CSFV). The SDS-PAGE analysis of the E2 protein of CSFV expressed in pET28a prokaryotic expression vector. M: protein molecular weight marker; Lane 1: uninduced recombinant pET28a containing E2 protein gene; lane 2: expression of protein in recombinant pET28a containing E2 protein gene after 2 h of 0.5 mM IPTG induction (A). Lane 3, 4, and 5: different fractions of purified recombinant E2 protein (B). Western blot analysis of recombinant E2 protein using polyclonal antibody raised in rabbits. Lane1: pET28a vector control; lane2: purified recombinant E2 protein lane3: CSFV purified virus control (C).

![Fig. 2.](image2.png) Fig. 2. The standard serial dilution method of determining observed CSFV ELISA antiserum titres from corrected absorbance with positive-negative threshold (PNT) baseline. N represents the PNT baseline while other lines show the different positive and negative serum samples.

![Fig. 3.](image3.png) Fig. 3. The relationship between the observed antibody titres of the serum samples obtained after serial dilution and their corresponding predicted antibody titres obtained from a single dilution ELISA at a 1:80 dilution.

The sensitivity, specificity and accuracy of the assay relative to the commercial diagnostic ELISA are shown in Table 1. The sensitiv-
ity, specificity and accuracy of recombinant E2 protein based ELISA was calculated 97.65%, 92.50%, and 95.24%, respectively.

CSFV shares antigenic epitopes with other pestiviruses such as border disease virus (BDV) and bovine viral diarrhea virus (BVDV) (Beer et al., 2007; Moenning, 2000). Although both BDV and BVDV are species specific, they may infect pigs making the diagnosis of CSFV difficult in field condition (Lin et al., 2000). Eradication of CSFV from swine requires serological methods which are rapid and simple to perform. Moreover, the test should clearly differentiate between CSFV and other pestiviruses. In addition, the test should also detect antibodies early during the CSFV infection. In view of the above facts expression of CSFV specific E2 protein is necessary to differentiate CSFV from BDV and BVDV. In the present study, the E2 protein of CSFV was expressed from a bacterial expression system and the purified protein was used as an antigen for diagnostic ELISA. Recombinant protein-based serological tests are considered to have higher sensitivity and specificity as the target antigen is immunodominant and devoid of any non-specific moieties present in whole cell preparations. We have successfully cloned and expressed the E2 protein of CSFV using the bacterial expression system. Although the actual biological activity of E2 being a glycoprotein in the bacterial expressed form may not be justifiable in the absence of post translational modification, its use in the diagnostics may have an added advantage. Our result showed high yield of E2 protein (70 mg/l) from bacterial culture suggests its efficient production. Our study showed that E2 protein based ELISA can be used for rapid and efficient screening of a large number of serum samples, especially during assessment of vaccination status of pigs involving large herd.

The sensitivity and specificity of the E2 protein based ELISA was 97.65% and 92.50%, respectively, relative to the commercial ELISA. The close correlation obtained between single serum and commercial ELISA titre showed similar trends in exposure to CSFV positive serum. However, the ELISA appears to be more sensitive than the commercial ELISA test and is able to detect antibody activity against CSFV in field samples that is not detectable by the commercial ELISA (Table 1). The ELISA reported in the paper using E2 protein would help to overcome some of the economic, technical and statistical constraints of using this assay as a rapid serological assay against CSFV infection in pigs. The recombinant E2 protein-based single serum dilution ELISA for the detection of antibodies developed in this study was shown to be sensitive, specific and accurate as compared to the available commercial ELISA. The study will help us to understand the use of viral surface protein in diagnostics of CSFV. These results demonstrate the potential benefit of a simple, specific ELISA for anti-E2 antibodies that may have diagnostic value for the pig industries. It will be interesting to explore the possibility of using other immunogenic proteins of CSFV for its diagnosis.

Table 1

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<tr>
<th>Single serum dilution ELISA</th>
<th>Commercial CSFV ELISA</th>
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<tbody>
<tr>
<td>+ve</td>
<td>166</td>
</tr>
<tr>
<td>-ve</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>170</td>
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Sensitivity: (166/170) × 100 = 97.65%. Specificity: (37/40) × 100 = 92.50%. Accuracy: (200/210) × 100 = 95.24%.

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References


