DISCUSSION

The North Eastern Region (NER) of India, owing to its unique geographical location sharing five international borders, bears a constant threat to India’s livestock for incursions of exotic as well as transboundary diseases. This region shares highly porous and sensitive frontiers with neighbouring countries. The NER shares approximately 4500 km international boundaries with Myanmar, Bangladesh, Bhutan, Nepal and China. Uncontrolled migration of animals from neighboring countries, therefore, can be great threat for spreading of emerging and transboundary diseases to this region. Among the livestock available in this region, pig rearing is an important agricultural activity in the eight NE states of India. Again pork is preferred meat for the non-vegetarian population. Pig population in NER constitutes nearly 40% of total pig population of India. However, limited infectious agents cripple this prosperous industry.

Classical swine fever (CSF), also known as hog cholera, is one of the most devastating disease of pigs causing substantial economic losses to the pig industry, particularly in NER and in India as a whole. Rapid, sensitive and specific laboratory diagnostic methods are therefore necessary to confirm and contain the outbreaks of CSF in pre-clinical phase. Phylogenetic analysis of CSF virus can depict the origin and spread of the virus prevailing in domestic pigs as well as wild boars in this region. Outbreaks recorded in different places in district Kamrup, Assam during the years 2012 and 2014 were analysed based on the partial nucleotide sequences of the E2, 5’NTR and NS5B genes. In addition, the kinetics of the cytokine response was
explored from whole blood of pigs vaccinated and infected with CSF virus in field condition, with a view of providing information regarding cytokine mediated mechanisms involved in infection and immunity.

**DETECTION OF CLASSICAL SWINE FEVER VIRUS**

Laboratory diagnostics of CSF plays a central role in the confirmation of an outbreak; rapid and precise detection of CSFV is crucial for disease containment. Virus isolation method, although a gold standard for disease diagnosis yet is labour intensive, requires extensive laboratory facilities, and is time consuming (de Smit et al., 2000). Other immunodiagnostic techniques like FAT (Anonymous, 1980; Wensvoort, 1986; Anonymous, 1996), antigen capture ELISAs (Dewulf et al., 2004) are handy to process large samples but are comparatively time consuming and less sensitive as there are cross-reactions with antibodies induced by other Pestiviruses. The nested PCR, an RT-PCR followed by a second PCR, is generally considered as the most sensitive *in vitro* method for the detection of CSFV, but the method is laborious due to the complexity of the sample preparation and therefore less suitable for processing large number of samples (Lowings et al., 1996a). Moreover, detection of amplified PCR products by gel-based systems bears the risk of cross-contamination since reaction tubes are required to open for RT step or at the end of the run for gel electrophoresis. Also, gel-based systems does not allow an exact quantification of genome copies and does not include tests for specificity (Belak and Thoren, 2001). Real-Time assay using SYBR Green may be non-specific and less accurate as intercalating dyes (SYBR Green I dye) generate fluorescence when bound to any dsDNA products including primer dimers. TaqMan assays for detecting the CSFV
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genome (Hoffmann et al., 2005; McGoldrick et al., 1999; Risatti et al., 2003, 2005a) proved to be practicable and robust for the diagnosis of pestiviruses and were used in a number of protocols (McGoldrick et al., 1998, 1999; Bhudevi and Weinstock, 2001, 2003; Risatti et al., 2003).

In the present study, specific oligonucleotide primers and the minor groove binding (MGB) fluorogenic probe described earlier (Hoffmann et al., 2005) were used for detection of CSFV nucleic acid from clinical samples. Such MGB probes form very stable hybrids with complementary DNA (Kumar et al., 1998). Fluorescence quenching is more efficient, giving increased sensitivity (Kutyavin et al., 2000; Afonina et al., 2002) and the higher melting temperatures (Tm) allows the design of significantly shorter probes (13–18 nucleotides) that are also more specific, especially if there is a mismatch in the MGB region of the duplex.

The one-step TaqMan™ Real-Time assay was performed in a single tube thus simplifying all pre- and post-PCR operating procedures. The use of one-step RT-PCR eliminated the additional manipulations that are generally required for a two-step reaction system and limits the risk of carry-over contamination. The assay detected CSFV, representing different phylogenetic groupings, but did not amplify viral RNA from related pestiviruses as the used oligonucleotide primers and the fluorogenic probe were specific to the highly conserved region within the 5’NTR of CSFV. Moreover, the assay detected the presence of the virus prior to the appearance of clinical disease and could be performed in 2 hrs or less, thus providing a rapid method for the diagnosis of CSF. In the present study a total of 210 whole blood samples were
screened, out of which 75 (35.7%) samples detected positive for CSFV by TaqMan™ Real-Time PCR.

Data presented here reconfirmed the observations of Risatti et al., (2003, 2005) that tonsil scraping, nasal swabs and blood are ideal samples for the detection of CSFV infection in early clinical phase. The early detection of CSFV suggested two potential uses in disease control for the assay: as a surveillance tool in areas free of the disease and as a screening assay for monitoring a disease outbreak in real time.

MOLECULAR CHARACTERIZATION OF CSFV

As genetic typing has become indispensable for epidemiological tracing of the isolates causing new outbreaks (Frias-Leporeau et al., 2002; Moennig et al., 2003), gel-based RT-PCRs will continue to be useful to check correct amplification before sequencing the products. The nucleotide sequencing data generated by the amplified RT-PCR products of CSFV genome were used for the comparative sequence analysis and interpretation of genetic relatedness among CSFV isolates.

Molecular epidemiology based on nucleotide sequence diversity is a useful tool for tracing virus spread and for developing disease control strategies. The phylogenetic analysis that were undertaken during the last decade demonstrated a link between genotype and geographical origin (Bartak and Greiser-Wilke, 2000). Therefore, it becomes possible to identify the possible origins for new outbreaks occurring in previously uninfected areas by genotyping a representative selection of viruses.
The present study described the characterization of RT-PCR amplicons of genomic E2, 5'NTR and 3'NS5B region of ten CSF field samples and their genotypic classification. The phylogenetic analysis of the generated 190 bp of E2, 150 bp of 5'NTR and 409 bp of NS5B nucleotide sequences was carried out from recent (years 2012–2014) CSF outbreaks originating from district Kamrup, Assam, India. Similar grouping of the field samples were observed based on phylogenetic analysis of E2, 5’NTR and NS5B sequence comparison. Genotype 2.2 and 2.1 were found in the studied cohort. It was revealed that the majority of the samples belonged to genotype 2.2 (80%), and a very few were grouped within genotype 2.1 (20%). All the sequences showed close similarity with the isolates from North East India. This findings were found to be similar to the recent reports of circulation of genotype 2.2 documented by Patil et al., (2010; 2011), Chakraborty et al., (2011) and Roychoudhary et al., (2014). Barman et al., (2014) reported similar findings of shifting of CSFV genotype from 1.1 to 2.2 in India between 2002–13. Barman et al., 2014 reported the circulation of genogroup 2 in wild boar populations from north-east part of India based on phylogenetic analysis of the partial E2 protein gene and 5’NTR of CSFV genome. In Europe, shifting of CSFV from genogroup 1–2 was reported in the 1980s (Paton et al., 2000). Similar findings were reported from China (Tu et al., 2001) and Taiwan (Deng et al., 2005). The prevalence of 2.2 genotype was detected in 2006 from states in the North, Central and Southern India (Barman et al., 2012). Present study revealed 20% prevalence of CSFV genotype 2.1 in district Kamrup, Assam. More genome sequence information on genotype 2.1 isolates would help in understanding the divergence of this emerging genotype of CSFV in India. Circulation of genotypes 2.1 in north-eastern part of India was indicated by Desai et al., (2010).
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Epidemiological mapping of the disease is essential to determine the strategy for control of such epidemic. For the effective control of CSF in India along with systematic vaccination, restriction of animal movement and sequestration of infected herd is utmost necessary. The top priority should be given to availability of diagnostic reagents and timely diagnosis of the disease. Though the present study was a preliminary epidemiological investigation involving only a few CSF field samples from Kamrup, Assam, it provided a baseline data about circulation of genotypes of CSFV in this part of Indian pig population. A comprehensive molecular epidemiology will provide avenue to select appropriate candidate for development of vaccine candidate to be used in CSF control program in India. The present findings however, warrant further study on CSF outbreaks taking large cohort populations encompassing all Northeast Indian states.

CYTOKINE RESPONSE FOLLOWING INFECTION AND VACCINATION WITH CSFV

CSFV pathogenesis might be ranging from life-threatening to asymptomatic, depending on the virulence of the virus strain and the immunocompetence of the host. CSF is a viral haemorrhagic disease which typically produces haemorrhage, thrombocytopenia and lymphoid depletion (Trautwein, 1988). The monocyte-macrophage has been identified as the main target cell for this virus, both in peripheral blood (Summerfield et al., 1998) and in the various lymphoid organs (Sato et al., 2000; Gomez-Villamandos et al., 2001). The virus targets immune cells, which are central in orchestrating innate and adaptive immune responses such as macrophages and conventional and plasmacytoid dendritic cells.
TNF-α and IL-6 are assumed to participate in the formation of haemorrhages characteristic of the acute form of CSF disease (Sanchez-Cordon et al., 2005a). TNF-α is a cytokine of innate immunity, the main effects of which include neutrophil activation, fever, synthesis of acute phase proteins and apoptosis of cells. TNF-α released during systemic viral infection is potentially a mediator for the induction of apoptosis in the lymphoid tissues (Zheng et al., 1995). IL-6 is a cytokine of innate immunity and regulates inflammation and transition from innate to adaptive immune responses. IFN-γ levels have been shown to be a good indicator of cell-mediated immunity (CMI) in pigs (Suradhat et al., 2001). Interleukin-10 is widely accepted to be a potent immunosuppressive cytokine that can strongly inhibit both innate and specific immune functions (Redpath et al., 2001). The cytokine IL-10 possesses pleiotropic effects in immunoregulation and inflammation, however it plays mainly an anti-inflammatory role in the immune system. In the present study valuable confirmation was established regarding association of different cytokines viz. TNF-α, IFN-γ, IL-10 and IL-6 in CSFV infection as well as in vaccination. Modulation in plasma cytokine concentration and cytokine gene expression were accessed using ELISA and quantitative RT-PCR respectively. Relative quantification (RQ=2^(-ΔΔCT)) of cytokine gene expression in vaccinated, unvaccinated-infected and vaccinated-infected groups were expressed as the fold change relative to the control (unvaccinated-infected) group.

**Vaccinated group vs Control group**

In the present study, no change was observed in the plasma cytokine concentration and mRNA expression levels of TNF-α and IL-6 in the vaccinated
group compared to the control group. This findings were found to be similar to the observation of Renson et al., (2013) who showed that TNF-α and IL-6 production were not significantly affected by CSFV vaccination.

In contrast to TNF-α and IL-6, the IFN-γ expression was upregulated by almost 15 folds compared to the control group; similarly plasma IFN-γ concentration increased (88 pg/ml vs 9.8 pg/ml for vaccinated and control group respectively) at 7 days post-vaccination (dpv). The present findings reaffirmed previous reports showing full protection and elevated number of IFN-γ producing cells as early as 6 days after vaccination with live CSFV vaccines (Suradhat et al., 2001, 2005; Tarradas et al., 2010). CSFV C-strain vaccine provide solid protection against challenge by 7 dpv which precedes the appearance of virus-neutralising serum antibodies (van Oirschot, 2003). Graham et.al. (2009, 2012) demonstrated the induction of robust IFN-γ response in pigs immunised with C-strain vaccine which were detectable from at least 9 dpv. The level of IFN-γ production can be used as an indicator for cell mediated immunity in pigs (Zuckermann et al., 1998; Suradhat et al., 2001).

In the present findings, IL-10 was found to be up-regulated by almost 7 folds compared to the control group; similarly plasma IL-10 concentration increased (29 pg/ml vs 7.8 pg/ml for vaccinated and control group respectively) at 7dpv. This moderate increase in IL-10 levels might be due to vaccination with live attenuated virus which propagate continuously in the host without causing disease. Moreover, IL-10, a pleiotropic cytokine, is involved in many different events having a complex role leading to different functions in the immune system (Li and Flavell, 2008),
including enhancement of B cell survival, proliferation and antibody production (Mosser and Zhang, 2008).

**Unvaccinated-infected group vs Control group**

In the present study, the level of IFN-\(\gamma\) in unvaccinated-infected group remained unchanged which is in agreement to the findings of Tarradas et al., (2010) who reported that in unvaccinated animals IFN-\(\gamma\) producing cells was below the detection level after CSFV infection. Further studies carried out by Graham et al., (2009, 2012) stated that unvaccinated animals that succumbed to CSFV infection had undetectable IFN-\(\gamma\) and delayed antibody responses and uncontrolled viraemia.

The present findings on IL-10 analysis showed almost 63 folds increase in expression level compared to the control group; similar pattern was observed in plasma IL-10 concentration by ELISA (343 pg/ml vs 7.8 pg/ml for unvaccinated-infected and control group respectively). This findings were in agreement with that of Suradhat et al., (2005) who showed that exposure to the virulent CSFV significantly increased IL-10 production in peripheral blood mononuclear cells of the CSF unvaccinated infected pigs. CSFV modulates T cells for cytokines secretion, such as IL-10, which might be a key cytokine in the immunosuppression observed after CSFV infection (Suradhat et al., 2005). The increased level of IL-10 post-infection was assumed to have a key role in immunosuppression observed during the course of CSF disease (Jamin et al., 2008). IL-10 facilitates immunosuppression and viral persistence during CSFV infection by inhibiting the activity of Th1 and NK cells (Blackburn and Wherry, 2007; Couper et al., 2008).
There was upregulation in expression level of TNF-α by almost 40 folds in the unvaccinated-infected group; similarly plasma TNF-α concentration was found to be 154 pg/ml in unvaccinated-infected group against 9.1 pg/ml in control group. This findings were supported by the reports of Graham et al., (2009) who noted significant levels of TNF-α in peripheral blood leukocyte from animals infected with CSFV from day 8 post-infection. Sanchez-Cordon et al., (2005a) reported that the maximum expression of TNF-α coincided with the largest number of cells showing signs of CSFV infection. TNF-α expression might contribute via apoptosis to the leukopenia seen clinically in CSFV-infected pigs (Choi et al., 2004).

The present findings indicated upregulation in IL-6 expression level by almost 17 folds in unvaccinated-infected group against control group; plasma IL-6 concentration was found to be 49 pg/ml vs 8.1 pg/ml in unvaccinated-infected group and control group respectively. This findings confirmed the observations made by Choi et al., (2004), Sanchez-Cordon et al., (2005a) and Borca et al., (2008) where they reported significantly higher levels of IL-6 expression in swine blood upon infection by virulent CSFV strain. IL-6 plays a role in modulating the immune response and in inducing apoptosis (Luster et al., 1999).

Jamin et al., (2008) investigated dendritic cell (DC) activation following an infection with CSFV in blood and secondary lymphoid organs of infected pigs in the early time post-inoculation (pi) period, and reported an increase in TNF-α, IL-10 and IL-6 level in serum. The uncommonly high levels of cytokines could play a role in disrupting immune system cells, by either inducing apoptosis and/or impairing DC maturation and T cell priming (Jamin et al., 2008).
Dead pigs vs Recovered pigs within Vaccinated-infected group

In the present study, dead pigs from vaccinated-infected group showed similar pattern of cytokine level for IL-10, TNF-α and IL-6 as was observed in the unvaccinated-infected group; which signified the fact that these animals were unprotected and therefore, immune-suppressed. Vaccine failure observed in dead pigs from vaccinated-infected group might be due to interference from maternal immunity and prolonged virus shedding from pigs infected with the CSFV genotype 2.2, rather than the immunogenicity of the vaccine itself (Suradhat and Damrongwatanapokin, 2003). Suradhat et al., (2003) reported that an increase in CSFV outbreaks might be due to the inappropriate timing of vaccination as well as the nature of the CSFV genotype 2.2. Since North East India is endemic for CSF, therefore, irregular vaccination and vaccination without proper serological monitoring of maternal immunity might be the reasons for vaccination failure to confer protection. This findings emphasised the importance of immunoprophylaxis, routine serological surveys, and strict biosecurity on farms within endemic areas, in order to prevent CSF outbreaks. However the recovered pigs, vaccinated pigs that survived the CSF infection, displayed a differential modulation in cytokine level within the vaccinated-infected group. It was observed that in the recovered pigs, vaccination attenuated the effects induced by CSFV infection on the production of IL-10, TNF-α and IL-6. In accordance with the present findings, Renson et al., (2013) demonstrated the interference of vaccines in the post-CSFV infection responses of IL-10, TNF-α and IL-6; which supported the fact that the production of these cytokines is related to CSFV pathogeny.
In the present study, IFN-γ expression was observed to be upregulated by almost 50 folds in the recovered pigs against only 19 folds increase in dead pigs; similarly plasma IFN-γ concentration was found to 335 pg/ml vs 101 pg/ml in recovered and dead pigs respectively. This findings signified the role of IFN-γ in controlling CSFV replication during infection. Successful vaccination might trigger the induction of IFN-γ producing cells in vaccinated animals prior to challenge with CSFV, which could be consistently detected also after CSFV infection (Tarradas et al., 2010). Similar findings were reported by Graham et al., (2009) who demonstrated elevated IFN-γ responses in vaccinated pigs 6 days post-challenge (11 days post-vaccination) following in vitro stimulation with C-strain virus. Sanchez-Cordon et al., (2005) reported significantly higher numbers of IFN-γ positive lymphocytes in pigs inoculated with the Alfort187 isolate of CSFV than in the untreated controls.

Neutralising antibody induced by vaccination with C-strain is a major protective mechanism, which is detected from 2–3 weeks post vaccination (Terpstra et al., 1990). Antibody (Ab) titers were determined in the present study and serum from dead pigs showed Ab titre of 1:20 to 1:40 while in recovered pigs antibody titre of above 1:80 was detected. Higher antibody titre might have provided major protection to the recovered animals, however, the data from this study suggested that the significantly higher level of IFN-γ produced post-vaccination could be correlated with the capacity of the vaccinated animals to control CSF infection. T cell IFN-γ responses which could be detected 7 days post-vaccination, might mediate protection through its antiviral activity in the absence of antibody (Suradhat et al., 2001; 2003). The correlation of antibody titres and cytokine patterns induced by vaccines could
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give a clear picture to reliably interpret the efficacy of vaccines with protective immunity.

In the present study, compared to recovered pigs, the difference in TNF-α expression level was found to be significantly elevated in dead pigs (p=0.001). Significantly higher expression of TNF-α in the dead pigs supported the finding of von Rosen *et al.*, (2013) that TNF-α levels might be involved in the immune responses during CSFV infection. The expression of IL-6 was significantly higher in dead pigs compared to recovered pigs (p=0.001). Luster *et al.*, (1999) demonstrated the role of IL-6 in modulating the immune response and in inducing apoptosis during viral infection. Significantly higher IL-10 expression level was present in dead pigs (p=0.001) compared to the recovered pigs. The increased level of IL-10 in dead pigs might play a key role in immunosuppression observed during the course of CSF disease (Suradhat *et al.*, 2005; Jamin *et al.*, 2008). Increased production of IL-10 induced by CSFV infection could be one of the strategies used by the virus to evade the host’s immune responses, thereby contributing to the unique clinical picture observed after CSFV infection. The IFN-γ expression in case of recovered pigs was found to be higher compared to dead pigs, the difference being statistically significant (p=0.001). These results pointed towards IFN-γ playing a role in the elicited protection against CSFV. IFN-γ production could be used to monitor the CSFV-specific immune response shortly after vaccination as the induction of IFN-γ production could be detected as early as 6 days following vaccination. Because type I IFN-γ is a signature cytokine of the T helper cell Th1-associated response, it is a useful indicator of cell-mediated immunity (CMI).
In the present study, the correlation between Th1 (IFN-γ) and Th2 (IL-10) was found to be statistically significant (p=0.001) by both Pearson Correlation and Spearman’s Rho. Significantly higher Th1:Th2 ratio (IFN-γ:IL-10) were present in vaccinated pigs that recovered from CSFV infection in comparison to vaccinated pigs that succumbed to CSFV infection. The present findings were supported by the study carried out by Renson et al., (2013) who reported attenuation of IL-10 gene expression induced by vaccination following CSFV challenge. The immunomodulatory cytokine IL-10 might inhibit the synthesis and release of other cytokines and contribute to reduced IFN-γ expression by T lymphocytes (Batista et al., 2004). Further inference could be drawn from the findings of Turner et al., (2002) who showed that the immunosuppressive cytokine IL-10 could suppress IFN-γ production in peripheral blood mononuclear cells (PBMCs) in pigs. The high expression of IL-10 observed in the case of dead pigs may be responsible for the reduced expression of IFN-γ, which in turn may prolong viral replication in pigs.

CSFV infection displays type 1 cell-mediated immune response in the early clinical stage, giving way to a type 2 immune response in the later stage which induces humoral response of CSF infection. As the present study was undertaken in the early clinical stage of CSF infection therefore dominant type 1 immunity was found to be elicited by vaccines. Thus the present findings on Th1:Th2 indicated that virtually all infections should be designed to skew the induced response toward a type 1 profile. Mechanisms to mediate vaccine-induced type 1 polarization should be undertaken by including the use of IFN-γ and IL-12 as vaccine adjuvants or by the inclusion of genes coding for IFN-γ, IL-12, or CpG motifs in DNA vaccines. In animal models, vaccines inducing type 1 immunity have been proven highly effective
at preventing infections, whereas vaccines inducing type 2 immunity increase susceptibility to infection (Cenci et al., 1990).

Taken together, in the present study, the TaqMan™ Real-Time PCR assay provided a rapid and specific diagnosis of CSFV from blood samples of pigs suspected for CSF virus infection in early clinical stage. Genotype 2.2 was the most prevalent genotype while genotype 2.1 was the only other genotype found in the studied cohort during the period December, 2012 to May, 2014 in district Kamrup, Assam. Comparing with past data, the present study indicated that there is shifting of CSFV genogroup from 1 to 2 in northeast India. Present findings on kinetics of the cytokine response suggested that IL-10 mediated immune suppression might play an important role in the pathogenesis of CSFV while IFN-γ response, which might mediate protection through its antiviral activity in the absence of neutralising antibody, is a useful indicator of cell-mediated immunity (CMI).