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Expression profiling of immune genes in classical swine fever vaccinated indigenous and crossbred piglets

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ABSTRACT

Classical swine fever is a highly contagious disease of pigs which courses from life-threatening to asymptomatic, depending on the virulence of the virus strain and the immune-competence of the host. The present study was undertaken to investigate the expression of immunologically important genes, viz. IFNα, IFNβ, SLA, SLA-2, SLA-DR, Ii, SLA-DM, CSK and JUN and to ascertain genetic group differences on the basis of humoral immune response. Blood samples were collected from 5 indigenous and 6 crossbred piglets at pre-vaccination and after 28th day of classical swine fever (CSF) vaccination. On 28th day, the competitive Enzyme Linked Immunosorbent Assay (cELISA) revealed poor humoral immune response (E2 antibodies) in indigenous piglets (84.80%) as compared to crossbred piglets (98.33%) in response to CSF vaccination. The expression level of genes was analyzed in three ways, viz. indigenous 28th day post-vaccination (28dpv) versus pre-vaccination, crossbred 28th day post-vaccination versus pre-vaccination and crossbred 28th day post-vaccination versus indigenous 28th day post-vaccination. The study showed that IFNα, IFNβ, SLA, SLA-2, Ii, SLA-DM, CSK and JUN were significantly upregulated in crossbred piglets than indigenous piglets at 28th day post-vaccination. But the SLA-DR was significantly downregulated in CSF vaccinated crossbred over indigenous piglets.

Key words: Classical swine fever virus (CSFV), Crossbred piglets, Immune genes, qRT-PCR

The research on breeding for disease resistance has always remained a challenging task for animal breeders due to limited scope of challenge in large animals and sporadic outbreak of disease. Hence, these circumstances provided the impetus for development of novel strategies to control livestock diseases. One strategy would be developing criteria for genetic selection of animals which show variation in immune response after vaccination. In many studies, a varied range of response, i.e. from poor to good responders has been observed even after immunization (Singh *et al*. 2016, Pathak *et al*. 2017). In general, these variations are ignored or considered as inevitable breed-to-breed and animal-to-animal variations. But there is possibility that at least some of the observed variation may be genetic. The understanding of genetics behind no response or low response or pathology at the other extreme may help in identifying new targets both for immunomodulators as well as for immunotherapeutics.

During the past few years, many outbreaks of swine fever have been recorded from different states of India, viz. Nagaland, Manipur, Tripura, West Bengal and Tamil Nadu (Rahman 2011). In the highly endemic areas, routine vaccination against CSF is the most common means used for prevention and control. The C strain, modified live vaccine (MLV) has been regarded as one of the most effective CSF vaccines that provides complete clinical and virological protection, i.e. sterile immunity, within a week of vaccination (Suradhat *et al*. 2001, Van Oirschot 2003). Hence transcriptional profile during CSF vaccination may facilitate the development of effective strategies for controlling classical swine fever. The animal yielding differential immune response may be basis for selecting breeding animal with natural resistance to swine fever. The identification of genes associated with immune response to vaccination may highlight new pathways that regulate the response to pathogens and be of more general importance. CSFV-induced apoptosis of thymocytes in the thymus (Saìnchez-Cordoìn *et al.* 2002) and of lymphocytes in the spleen (Saìnchez-Cordoìn *et al.* 2005) is considered to be related to the increased expression of cytokines in monocytes–macrophages, whilst apoptosis of T lymphocytes during classical swine fever (CSF) is associated with an increase in CD49d, major histocompatibility complex II and Fas gene expression (Summerfield *et al.* 1998). Again, the use of vaccine virus

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(vaccination) would reduce the use of animal in challenge study (live virus) and during natural course of vaccination the response to vaccine can be utilized in formulation of breeding plan of livestock for specific disease. Therefore, in present study, gene expression profile of immunologically important genes were investigated in indigenous and crossbred piglets before and after CSF vaccination.

MATERIALS AND METHODS

Experimental animals: The experimental procedures in the present study were approved by Institute Animal Ethics Committee. Indigenous and crossbred piglets maintained at Indian Veterinary Research Institute, Izatnagar under AICRP were included in present investigation. Piglets were checked for maternally derived antibodies (MDA) before CSF vaccination using c-ELISA which was found nil. The C strain Lapinized vaccine was used for vaccination.

Collection of blood samples and ethics statement: The permission for blood collection was taken from Institute Animal Ethics Committee as per guidelines. For RNA isolation, about 5 ml of blood was collected from 11 randomly selected healthy piglets (5 indigenous and 6 crossbred) in heparin coated vacutainer under sterile conditions at two different time points, i.e. on the day of vaccination and 28 days post-vaccination (dpv). After collection of blood, the vacutainer tubes were tightly capped and shaken gently to facilitate thorough mixing of blood with the anticoagulant. The vacutainer tubes were then immediately transported to the laboratory in icebox.

For competitive Enzyme Linked Immunosorbent Assay (cELISA), about 3 ml of blood was collected from same piglets in plain vacutainers or vacutainers without any anticoagulant at different time points, i.e. just before day of vaccination and 28 dpv to get hyper immune serum. Vacutainers were then kept at slanting position for 2 h on ice and then kept overnight at 4°C in the refrigerator for better yield of serum. Isolated serum was collected next day.

RNA isolation and cDNA synthesis: RNA was isolated from whole blood of indigenous and crossbred piglets during both pre- and post-vaccination stage by Trizol method. The purity of the RNA was assessed by Nanodrop measuring the absorbance of RNA solution at 260 nm and 280 nm. The RNA samples showing the OD_{260}:OD_{280} value between 1.9–2.2 were considered as good quality and used for further analysis. The Reverse transcription of total RNA was carried out using QuantiTect Reverse Transcription kit for cDNA synthesis according to the manufacturer's instructions. The cDNA product was stored at –20°C.

cELISA for measuring humoral response: cELISA was performed in pre- and post-vaccination serum of all 11 piglets. The PRIOCHECK CSFV Ab Serum ELISA kit was used to detect E2 antibodies in response to CSFV vaccination in individual serum using prescribed protocol.

Real time based analysis of transcript abundance: Real time PCR (RT-PCR) was performed using CFXTM 96 BioRad real time machine. GAPDH was used as an endogenous control. The primer sequences of genes used in the study are given in Table 1. All the samples were run in triplicates. The amplification was carried out in 20 ml volume reaction mixture containing 10 ml of 2× master mix (Qiagen SYBR Green qPCR Master Mix), 1 ml (10 pmol) each of gene specific forward and reverse primer, 2 ml cDNA template and 6 ml nuclease free water. Negative

Table 1. Primer details viz. Gene ID, sequence, amplicon size and annealing temperature of different genes used in RT-PCR

Gene	Gene ID	Primer sequence	Amplicon size (bp)	Annealing temperature (°C)
IFNα	M28623	F TGGGAGATCGTCAGGGCAG R GACATGGCAGAACAGGAGG	155	57
IFNβ	EF104599	F GGACAGTTGCCTGGGACTC R GGAGCATCTCGTGGATAATC	128	57
SLA	100157996	F CGGGTCAGTTCACCTACGAT R GATCAGTGCAATGCCTCTCA	143	57
SLA-2	AF464005	F CGCACAGACTTTCCGAGTG R GTCTGGTCCCAAGTAGCAG	110	57
SLA-DR	DQ883222	F GTGTGCGACGGAATCTATAAC R GAGCATGAGCCCTAAGAGAC	258	57
Ii	AB116558	F GCAACGCCACCAAGTACGG R AAGAGCCACTGACGCAGCC	185	57
SLA-DM	100135050	F TCTCCCATTTGGCTACAACC R GAGTCGCCACAGACACAGAA	147	57
CSK	100154110	F GACGTGTGGAGTTTCGGAAT R AGGTGCCAGCAGTTCTTCAT	173	57
JUN	396913	F CCCAAGATCCTGAAGCAGAG R GATGTGCCCGTTACTGGACT	174	57
GAPDH	AF017079	F TGGTGAAGGTCGGAGTGAAC R GAAGATGGTGATGGGATTTC	225	57

control was included for the RT-qPCR assay. In negative control (NTC), cDNA was not added. The thermal profile used was 95°C for 15 min then 40 cycles of 95°C for 15 sec, 57°C for 30 sec, and 72°C for 30 sec with fluorescence recording at the end of each cycle, followed by denaturation of products from 65°C to 95°C with fluorescence recording throughout the step.

Statistical analysis: Expression level of genes was analyzed in three ways—indigenous post-vaccination versus pre-vaccination, crossbred post-vaccination versus pre-vaccination and vaccinated crossbred versus indigenous. The relative expression of each sample was calculated using the 2-ΔΔCt method with control group as calibrator (Schimittgen and Livak 2008). One way ANOVA was done in JMP9 (SAS Institute Inc, Cary, USA) and differences between groups were considered significant at P<0.05.

RESULTS AND DISCUSSION

The piglets used for blood collection were screened for CSFV maternally derived antibodies (MDA) and all the animals had 0% (nil) percentage inhibition (PI) on day of vaccination. Again cELISA was done on 28th day after CSF vaccination to detect the presence of CSFV specific antibodies in indigenous and crossbred piglets. The mean PI in indigenous piglets (5) was 84.8% and in crossbred piglets (6) was 98.33% and our findings were in agreement with findings of Singh *et al.* (2016). In order to gain more insight into the mechanisms of host genes associated immune response after CSF vaccination, quantification of mRNA transcript of immunologically relevant genes was done for ascertaining genetic group differences.

The expression level of immunologically important genes was measured in terms of log2 fold change in three possible different combinations in response to Classical Swine Fever vaccination between two different genetic groups (Fig. 1). The findings are being enumerated under heading of different genes under investigation.

IFNα gene: The expression of IFNα gene was non-significantly upregulated (0.783 fold) at 28dpv in vaccinated indigenous piglets than unvaccinated indigenous piglets. However, in crossbred piglets, the IFNα gene was significantly (P≤0.01) upregulated (10.042 fold) at 28dpv in vaccinated piglets over unvaccinated piglets. Further, expression of IFNα gene in vaccinated crossbred piglets was significantly (P≤0.01) upregulated (8.622 fold) than vaccinated indigenous piglets at 28dpv.

IFNβ gene: The expression of IFNβ gene was significantly (P≤0.01) downregulated (–4.847 fold) at 28dpv in vaccinated indigenous piglets than unvaccinated indigenous piglets. But, in crossbred piglets, the expression of IFNβ gene was non-significantly upregulated (2.393 fold) at 28dpv in vaccinated piglets over unvaccinated piglets. It was observed that in crossbred vaccinated piglets, the IFNβ gene was significantly (P≤0.01) upregulated (7.876 fold) than vaccinated indigenous piglets at 28dpv.

SLA gene: The expression of SLA gene was non-

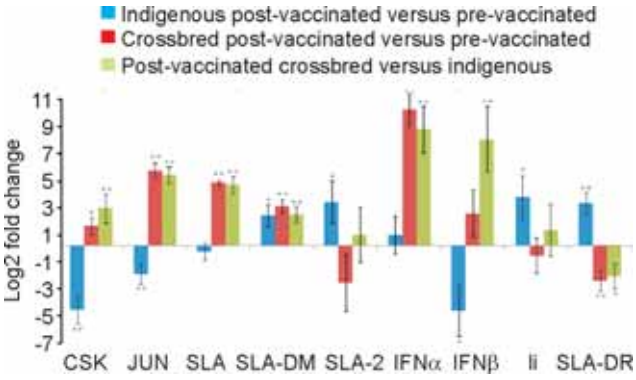

Fig. 1. Log2 fold change of mRNA in indigenous post-vaccinated verses pre-vaccinated, crossbred post-vaccinated verses pre-vaccinated and post-vaccinated crossbred *verses* indigenous piglets.

significantly downregulated (–0.442 fold) at 28dpv in vaccinated indigenous piglets than unvaccinated indigenous piglets. But, in crossbred piglets, the expression of SLA gene was significantly (P≤0.01) upregulated (4.665 fold) at 28dpv in vaccinated piglets over unvaccinated piglets. It was observed that in crossbred vaccinated piglets, the SLA gene was significantly (P≤0.01) upregulated (4.511 fold) than vaccinated indigenous piglets at 28dpv.

SLA-DM gene: The expression of SLA-DM gene was significantly (P≤0.01) upregulated (2.214 fold) at 28dpv in vaccinated indigenous piglets than unvaccinated indigenous piglets. Also, in crossbred piglets, the SLA-DM gene was significantly (P≤0.01) upregulated (2.917 fold) at 28dpv in vaccinated piglets over unvaccinated crossbred piglets. Further, expression of SLA-DM gene in crossbred vaccinated piglets was significantly (P≤0.01) upregulated (2.354 fold) than vaccinated indigenous piglets at 28dpv.

SLA2 gene: The expression of SLA2 gene was significantly (P≤0.01) upregulated (3.237 fold) at 28dpv in vaccinated indigenous piglets than unvaccinated indigenous piglets. But, in crossbred piglets, the expression of SLA2 gene was non-significantly downregulated (–2.753 fold) at 28dpv in vaccinated piglets over unvaccinated piglets. It was observed that in vaccinated piglets, the SLA2 gene was non-significantly upregulated (0.830 fold) than vaccinated crossbred piglets at 28dpv.

Ii gene: The Ii gene was significantly (P≤0.05) upregulated (3.597 fold) at 28dpv in vaccinated indigenous piglets than unvaccinated indigenous piglets. However, in crossbred piglets, the Ii gene was non-significantly downregulated (–0.709) at 28dpv in vaccinated piglets over unvaccinated piglets. Further, expression of Ii gene in crossbred vaccinated piglets was non-significantly upregulated (1.148 fold) as compared to vaccinated indigenous piglets at 28dpv.

SLA-DR gene: The SLA-DR gene was significantly (P≤0.01) upregulated (3.141 fold) at 28dpv in vaccinated indigenous piglets, than unvaccinated indigenous piglets. But, in crossbred piglets, the SLA-DR gene was significantly (P≤0.01) downregulated (–2.587 fold) at 28dpv

in vaccinated piglets over unvaccinated piglets. Further, it was observed that in crossbred vaccinated piglets, expression of SLA-DR gene was significantly (P≤0.01) downregulated (–2.272 fold) than vaccinated indigenous piglets at 28dpv.

CSK gene: The expression of CSK gene was significantly (P≤0.01) downregulated (–4.703 fold) at 28dpv in vaccinated indigenous piglets than unvaccinated indigenous piglets. But, in crossbred piglets, the expression of CSK gene was significantly (P≤0.05) upregulated (1.437) at 28dpv in vaccinated piglets over unvaccinated piglets. It was observed that in crossbred vaccinated piglets, the CSK gene was significantly (P≤0.05) upregulated (2.773 fold) than vaccinated indigenous piglets at 28dpv.

JUN gene: The JUN gene was significantly (P≤0.01) downregulated (–2.068 fold) at 28dpv in vaccinated indigenous piglets than unvaccinated indigenous piglets. However, in crossbred piglets, the CSK gene was significantly (P≤0.01) upregulated (5.551 fold) at 28dpv in vaccinated piglets over unvaccinated piglets. Further, it was observed that in crossbred vaccinated piglets, expression of CSK gene was significantly (P≤0.01) upregulated (5.224 fold) than vaccinated indigenous piglets at 28dpv.

Type I interferon (IFN-α/β) are one of the most important and potent antiviral cytokines and modulators of the adaptive immune system. Type I IFN plays a major role in the CD8 T-cell response to viral infection, and its effects are on both the APCs and on the T cells (Welsh *et al.* 2012). It can provide a major co-stimulatory effect in its own right by binding to the IFN1R on CD8 T cells and greatly augmenting their proliferation (Curtsinger *et al.* 2005, Kolumam *et al.* 2005, Thompson *et al.* 2006). Reports suggested a strong correlation between lymphopenia and the IFNα levels in sera from CSFV-infected pigs (Summerfield *et al.* 2006, Jamin *et al.* 2008). Renson *et al.* (2010) reported strong up-regulation of ISG (Interferon Stimulated Genes) in PBMC and down-regulation of IFNα in these same cells on day 1 (D1), day 2 (D2) and day 3 (D3) of post-infection with two CSFV strains (Eystrup strain and Paderborn strain). It was also reported that Type 1 IFN upregulates expression of both MHC and co-stimulatory molecules greatly affecting the initiation of T-cell responses (Montoya *et al.* 2002). In CSFV infection, host anti-viral Type I IFN, such as alpha-interferon (IFN-α), was suppressed in infected dendritic cells, and some other host cytokines including IL-6, IL-10, IL-12, and TNF-α were also not induced (Chen *et al.* 2012). Type I IFN is known to promote the induction of apoptosis in infected cells (Tanaka *et al.* 1998), however, IFNα had also been reported to induce death in various non-infected cells (Thyrell *et al.* 2002, Jiang *et al.* 2005).The present study showed that IFN-α and IFN-β were significantly upregulated at 28dpv in crossbred piglets, which indicates better antibody production of crossbred pigs than indigenous pigs. The IFN α/β mRNA upregulation might reflect a loss of N^{pro} expression in macrophage of crossbred pigs**.** The absence of N^{pro} also correlated with the loss of the capacity of the virus to interfere with dsRNA-mediated apoptosis and IFN- α/β induction (Ruggli *et al.* 2005). Studies suggest that the high and prolonged IFN-I responses found in animals are correlated with the lymphopenic syndrome and disease severity (Summerfield *et al.* 2006, Ruggli *et al.* 2009, Renson *et al.* 2010).

The highly polymorphic swine leukocyte antigen (SLA) genes have been repeatedly shown to influence swine immune traits, disease resistance, vaccine responsiveness and tumor penetrance. In our study, we investigated the expression of five MHC genes (SLA, SLA-DM, SLA2, SLA-DR, li) on 28dpv in both crossbred and indigenous piglets. We found that SLA, SLA-DM, SLA2 and li genes were significantly upregulated in crossbred piglets compared to indigenous piglets. Therefore, our results revealed that crossbred piglets had better humoral immune response than indigenous piglets. The global transcriptional profiles of peripheral blood mononuclear cells during CSFV infection have shown that cellular genes present a low level of up- and down-regulation *in vivo* (Li *et al.* 2010). The studies showed that certain immune response genes, SLA-2 (MHC class I), TAP1, SLA-DR (MHC class II), Ii, CD40, CD80, and CD86, were down-regulated *in vivo* from 6 to 9 dpi in Large White × Landrace piglets. Feng *et al.* (2012) also reported changes in the mRNA expression at various time points (1, 3, 12, 24, 36 and 48 hpi) of host immune genes associated with the MHC antigen presentation pathway, such as MHC class I swine leukocyte antigen 2 (SLA-2), MHC class II swine leukocyte antigen DR (SLA-DR) and MHC class II associated invariant chain (Ii). They reported that immune response genes were generally down-regulated as a result of CSFV infection, and the expression of SLA-2, SLA-DR, Ii and CD80 was significantly decreased.

The CSK gene is one of the important genes in B cell receptor pathways and was significantly unregulated in crossbred pigs than indigenous pigs. CSK acts by suppressing the activity of the Src family of protein kinases by phosphorylation of Src family members at a conserved C-terminal tail site in Src (Nada *et al.* 1993, Chong *et al.* 2006). Previous study showed that over-expression of CSK in macrophages resulted in reduced TNF-α, IL-1α, IL-6 and no production in response to LPS (Iwabuchi *et al.* 1997). However, another study reported that over-expression of CSK enhanced TLR4 and TLR9 signaling and caused a significant induction of tyrosine phosphorylation of multiple cellular proteins (Aki *et al.* 2005). In addition, JNK and AP-1 were up-regulated by LPS stimulation in CSK over-expressing cells (Kizaki *et al.* 2001). Therefore, over-expression of CSK appears to up-regulate the Src family kinases or other tyrosine kinases in macrophages. c-Jun NH2-terminal kinases (JNKs) are a group of mitogen-activated protein (MAP) kinases that participate in signal transduction events mediating specific cellular functions. The present study showed that JUN gene was significantly upregulated at 28 dpv in crossbred piglets compared to indigenous piglets. Activation of JNK is regulated by

phosphorylation in response to cellular stress and inflammatory cytokines. Motameni (2007) reported that JNK1 regulates the response to TLR1/2 ligands and suggest a positive feedback loop that may serve to increase the immune response to the spirochete. It was also observed that deficiency of JNK genes exhibit severe defects of T cell–mediated immune responses in mice (Dong *et al.* 1998, Yang *et al.* 1998, Sabapathy *et al.* 1999). Dong *et al.* (1998) also reported increased production of IL-4, IL-5, and IL-10 in JNK1 knockout mice.

Hence, the present study indicated the expression level of immunologically important genes, viz. IFNα, IFNβ, SLA, SLA-2, Ii, SLA-DM, CSK and JUN were significantly upregulated in crossbred than indigenous piglets on 28 day post-vaccination of CSFV. This result inferred that crossbred piglets have better humoral immune response to CSFV than indigenous piglets. Therefore, identification of genes associated with immune response to vaccination may highlight new pathways that regulate the response to pathogens and be of more general importance.

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