



Expression profiling of miR-146a-3p and miR-1343 with their target genes after classical swine fever vaccination

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Classical swine fever is one of the most dreaded and devastating viral disease of pig causing high morbidity and mortality worldwide with widespread economic implications. The disease is caused by a *Pestivirus* named classical swine fever virus (CSFV), an enveloped, single-stranded, positive RNA virus, approximately 12.3 kb in length (Risatti *et al.* 2003). The disease courses from life-threatening to asymptomatic, depending on the virulence of the virus strain and the immunocompetence of the host. The virus targets immune cells, which are central in orchestrating innate and adaptive immune responses such as macrophages and conventional and plasmacytoid dendritic cells (Summerfield and Ruggli, 2015). The miRNAs are an abundant class of highly conserved small (18–25 nt long) non-coding RNAs, regulating the gene expression by binding to the 3' untranslated region (UTR) of target mRNAs, which presents an entirely new approach to post-transcriptional regulation of gene expression (Roberts *et al.* 2011a). Role of miRNAs as gene regulators can be attributed to the fact that they constitute 1% of the total genes in the animals and regulate approximately 30% of their protein coding genome (Filipowicz *et al.* 2008). Cellular miRNA expression is profoundly influenced by viral infection, which can be attributed to both host antiviral defenses and viral factors altering the cellular environment. miRNAs can target viral RNAs during infections, resulting in promotion or inhibition of virus replication (Li *et al.* 2014, Mizuguchi *et al.* 2015) or a new pathway to alter the virus life cycle (Chen *et al.* 2011). In present investigation two

miRNA miR-146a-3p and miR-1343 were selected for investigating the expression profiling before and after CSF vaccination. Also expression profile of target gene of each miRNA was investigated on 7th day post vaccination as compared to unvaccinated control pigs.

All procedures involving animals were carried out as per the Indian Veterinary Research Institute animal ethics committee. A total of six synthetic Landilly crossbred pigs (*Landrace* × *Desi*), maintained at All India Coordinated Research Project (AICRP) unit, Indian Veterinary Research Institute (IVRI), Izatnagar were selected for present study. After weaning at three months of age, the selected pigs were vaccinated with IVRI strain of lapinized CSF virus after ensuring the sero-negativity for maternally derived antibody (MDA). About 5 ml of blood was collected from each crossbred pigs on 0 and 7 dpv in heparin coated vacutainer under sterile conditions. The whole blood sample was diluted with PBS at a ratio of 1:1 and layered carefully on 3 ml of histopaque-1077 Sigma (3:1) and centrifuged at 2,500 rpm at room temperature without brake for 45 minutes. In a separate tube the interphase layer which is rich in peripheral blood mononuclear cells (PBMCs) was transferred and was washed three times with PBS (1×) at 2,500 rpm for 15 min at 4°C. The final pellet was suspended in RPMI-1640 with 10% fetal calf serum with centrifugation at 2,500 rpm for 15 minutes at 4°C. Isolated PBMC were pooled in a separate Eppendorf tube and 1.5 ml of TRIzol Ambio reagent was added into it. The pooled PBMC samples were then stored at –80°C for total RNA isolation.

Two miRNA namely ssc-miR-146a-3p and ssc-miR-1343 were selected for expression study which were reported to be differentially expressed during miRNA Seq analysis in our laboratory (Sailo *et al.* 2019). Total RNA including small RNA were isolated from pooled PBMC samples of 0 and 7 dpv using mirVana™ miRNA isolation kit (Invitrogen). The cDNA is reverse transcribed from total RNA samples using a miRNA-specific, stem-loop RT primer of ssc-miR-146a-3p, ssc-miR-1343 and U6snRNA from TaqMan® MicroRNA Reverse Transcription Kit as per the prescribed protocol. The cDNA product was stored

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at -20°C . The qRT-PCR was performed using a standard TaqMan miRNA assay on an Applied Biosystems 7500 fast Sequence Detection System. The relative expression of each sample was calculated using the $2^{-\Delta\Delta\text{CT}}$ method with control group as calibrator (Schmittgen and Livak 2008).

Target genes of ssc-miR-146a-3p and ssc-miR-1343 were predicted using TargetScan tool (Aggarwal *et al.* 2015). The miRNA–target genes network was created based on the expression profile of target genes and miRNAs using Cytoscape (ver.3.1.1) (Shannon *et al.* 2003). Functional annotation was performed to find out the gene ontology (GO) terms enriched using target genes of miRNA governed by them in ClueGO (ver. 2.1.4) (Bindea *et al.* 2009) in Cytoscape (ver. 3.1.1).

The *CD86* (target gene of ssc-miR-146a-3p) and *IFIT1* (target gene of ssc-miR-1343) were selected for quantification by real time PCR. Target genes were selected based on their fold change, gene expression on particular time points and biological functions in regulation and activation of immune system process. RNA was isolated from pooled PBMC samples of 0 and 7 dpv by Trizol method. The Reverse transcription of total RNA was carried out using Revert Aid First Strand cDNA Synthesis Kit for cDNA synthesis according to the manufacturer's instructions. The cDNA product was stored at -20°C .

The primers were designed using PrimerQuest tool software (<https://www.idtdna.com/Primerquest>) and real-time PCR was performed on Applied Biosystems 7500 Fast real time PCR using $2 \times$ SYBR Green Master mix. *GAPDH* was used as an endogenous control. The primer sequences of target genes used in the study for validation are given in table 1. All the samples were run in triplicates and the relative expression of each sample was calculated using the $2^{-\Delta\Delta\text{CT}}$ method with control group as calibrator (Schmittgen and Livak 2008).

The expression of immunologically important DE miRNAs and their target genes were measured in terms of log2 fold change at two different time points (0 and 7 dpv) to study the differential expression in response to classical swine fever vaccination (Fig. 1). It was observed that the expression of ssc-miR-146a-3p was upregulated with a fold change of 1.243 in qRT-PCR validation, which were also found to be upregulated in miRNA sequencing with a fold change of 2.818 on 7 dpv compared to unvaccinated crossbred pigs (Sailo *et al.* 2019). However, it was observed that ssc-miR-1343 was downregulated with a fold change

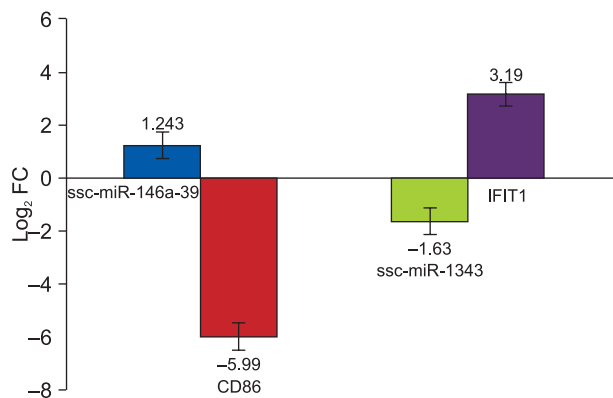


Fig. 1. qRT-PCR validation of ssc-miR-146a-3p, ssc-miR-1343 and their target genes on 7 dpv versus 0 dpv.

of -1.63 in qRT PCR, which were further found to be downregulated in miRNA sequencing analysis with a fold change of -0.713 on 7 dpv compared to unvaccinated crossbred pigs (Sailo *et al.* 2019).

It was observed that there were 378 and 398 target genes regulated by ssc-miR-146a-3p and ssc-miR-1343. The highly enriched common GO terms and pathways regulated by ssc-miR-146-3p consist of immune response-activating signal transduction, regulation of B cell activation, T-cell differentiation, positive regulation of leukocyte mediated immunity, leukocyte mediated cytotoxicity and regulation of myeloid leukocyte differentiation. The enriched common GO terms and pathways regulated by ssc-miR-1343 include regulation of defense response to virus by host, regulation of innate immune response, positive regulation of adaptive immune response and negative regulation of immune effector process.

It was observed that *CD86* (target gene of ssc-miR-146a-3p) was downregulated with fold change of -5.99 on 7 dpv compared to unvaccinated pigs. It was found that *IFIT1* was upregulated in qRT-PCR with fold change of 3.19 and on 7 a dpv compared to unvaccinated pigs. It was found that upregulation of ssc-miR-146a-3p on 7 versus 0 dpv potentially regulates the expression of *CD86*, *TNFAIP3*, *LAT2*, *TRAF6*, *RIPK1*, *IL7R*, *ZFP36L2*, *BST1*, *RABGEF1*, *THY1*, *THY1*, *GZMB*, *CLEC7A*, *CRTAM*, *MEF2C*, *TFE3*, *RPS6KA3*, *STX4*, and *CD3D* genes that are involved in immune response against pathogens. The miR-146 play crucial role in control of Toll-like receptor and cytokine signaling through a negative feedback regulation loop

Table 1. Primer sequence of target genes

miRNAs	Target gene	Primer sequence	Annealing Temperature ($^{\circ}\text{C}$)	Amplicon size (bp)
<i>GAPDH</i> (endogenous control)		F ACATGGCCTCCAAGGAGTAAGA R G ATCGAGTTGGGGCTGTGACT	60	106
ssc-miR-146a-3p	<i>CD86</i>	F CTACACAAGGCTACCCAGAAC R GGAAGAGACACCCTGATTGATAC	60	142
	<i>IFIT1</i>	F TCAGAGGTGAGAAGGCTGGT R GCAAGTGTCCTCACCTTGT	60	108

involving down-regulation of *IL-1* receptor-associated kinase 1 and TNF receptor-associated factor 6 (*TRAF6*) protein levels (Taganov *et al.* 2006). Higher expression of miR-146a was reported in effector memory and central memory than in naive human CD8⁺ T lymphocytes (Curtale *et al.* 2010). The results of real-time PCR revealed that target genes of ssc-miR-146a-3p *CD86* gene was downregulated on 7 dpv compared to unvaccinated crossbred pigs. However, *CD86* gene is a costimulatory molecule which interacts with *CD28* for T-cell activation and survival and with *CTLA4* for immune regulation. Downregulation of *CD86* was reported in monocyte derived macrophages with a fold change of -2.579 in indigenous pigs compared to crossbred pigs at 60 hpi, inhibiting the dendritic cell maturation which indicated the intact activity of Erns in virulent IVRI strain of CSFV (Sah 2017). On contrary, it was observed that *CD86* expression on HCV specific CD8⁺ T cells was linked to effective TCR stimulation with sufficient IL-2 signaling (Radziewicz *et al.* 2010).

Downregulation of ssc-miR-1343 on 7 versus 0 dpv potentially modulates the expression of *IFIT1*, *TGFB1*, *SASH3*, *KLRK1*, *SOCS5*, *DLG1*, *TLR4*, *OTUD7B*, *MED1*, *MUL1*, *STX7*, *SART1*, *PYCARD*, *SERPING1*, *NRARP* and *LEF1* that play important role in regulation of defense response to virus by host. It was reported that miR-1343-3p reduces the expression of *TGF-β* receptor-1, which induces angiogenesis through vascular endothelial growth factor (VEGF)-mediated apoptosis (Ferrari *et al.* 2009). It was found that miR-1343 reduces the expression of both *TGF-β* receptor 1 and 2 by directly targeting their 3'-UTRs in fibrotic lung disease (Stolzenburg *et al.* 2016). The results of real-time PCR revealed that target gene of ssc-miR-1343, viz. *IFIT1* genes was upregulated on 7 dpv compared to unvaccinated crossbred pigs. Induction of *IFIT* transcription is triggered by many stimuli, usually in context of viral and bacterial infections. *IFIT1* and *IFIT2* are involved in a nonspecific antiviral program through their direct interactions with eIF3, which subsequently suppresses more than 60% of translation in cells and viruses during protein synthesis (Hui *et al.* 2005). Evidence suggest that over-expression of *IFIT1* could inhibit virus triggered activation of IFN-β, NF-κB and IRF3.

SUMMARY

The expression profiling of the miRNAs, ssc-miR-146a-3p and ssc-miR-1343 in the PBMCs of classical swine fever (CSF) vaccinated crossbred pigs were investigated on 7 days post vaccination (7 dpv) as compared to unvaccinated pigs. It was observed that ssc-miR-146a-3p was up-regulated (1.243 Log₂ FC) and ssc-miR-1343 was down-regulated (-1.63 Log₂ FC) on 7 dpv compared to unvaccinated crossbred pigs which were in concordance with earlier report of miRNA Seq expression profiling. Two target genes, (*CD86* for ssc-miR-146a-3p and *IFIT1* for ssc-miR-1343) were validated by qRT-PCR and were also found to be in concordance with miRNA expression profile. The *CD86* was downregulated with log2 fold changes -5.99, whereas

the *IFIT1* was upregulated with log2 fold changes 3.19 at 7 dpv. Both of these miRNA was actively involved in cell mediated immune response at 7dpv after CSF vaccination. The CSF vaccine virus triggered the expression of host miRNAs and its target mRNA and enriched immune system processes/pathways.

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