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CD163 expression in monocyte derived macrophages of Landrace and Ghurrah breeds confers susceptibility to Classical swine fever virus infection

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Abstract
Classical swine fever (CSF) is a highly infectious and usually fatal disease of swine caused by the classical swine fever virus (CSFV), a member of the pestivirus genus and the Flaviviridae family. CSFV is a spherical viral particle with a 40-60 nm in size and its nucleocapsid having positive-strand RNA genome of 12.3 kb. In our present study we identified CD163 receptor expression in Classical swine fever virus infected porcine monocyte derived macrophages among two breeds of swine (Ghurrah and Landrace). The expression of CD163 which is the receptor of monocytes/macrophages was found to be upregulated in Landrace as compared to Ghurrah. Because, there is correlation between permissiveness of CSF virus with the CD163 receptor expression, the viral copy number was assessed by real-time PCR in these two breeds. So, we found high viral load in Landrace which signifies more virus permissiveness or increased infectiousness in mature macrophages of Landrace. This is the first study for the comparison of CD163 receptor expression with CSFV viral load in Ghurrah and Landrace.

Keywords: Classical swine fever virus, Flaviviridae, CD163 receptor, monocyte derived macrophages, viral copy number

Introduction
Classical swine fever (CSF) is a highly contagious disease of swine and wild hog which is characterized by high fever, diarrhea, hemorrhage, leukopenia, thrombocytopenia, immunosuppression, fertility problems, and high mortality. It is a deadly disease that causes significant economic losses due to the death of valuable livestock. As a result, the causal agent of CSF, classical swine fever virus (CSFV), is recognised as a disease by the World Organisation for Animal Health (OIE) [1]. Due to the stringent immunisation programmes, modifications in the epidemiological form and clinical signs of CSF have been noted. Major epidemics are now scarce, although sporadic epizootics with chronic illnesses, still often atypical cases of the disease occur. There have also been reports of abortions, mummifications, stillbirths, abnormalities, and the birth of immature and weak piglets [2].
The etiological agent, CSF virus is a member of the genus pestivirus and belonging to the Flaviviridae family. Shape of virus particle is spherical/icosahedral with a 40–60 nm in diameter comprising of a lipid-enveloped nucleocapsid protein packing a 12.3 kb length positive-sense polarity RNA genome [3]. The CSFV genome is comprised of one single open reading frame (ORF) that encodes around 3900-amino-acid polyprotein that is co- and post-translated into 11–12 final individual proteins (NH2-Npro-C-Erns-E1-E2-p7-NS2-NS3- S4A-NS4B-NS5A-NS5B-COOH) by cellular and viral protease enzymes[4]. Untranslated regions (UTRs) flanking the ORF at 5' and 3' regions are largely conserved within virus isolates [5]. In both natural and experimental scenarios, it has been observed to elude the host immune system and to establish chronic infection [6]. Particularly in CSF virus infection, host anti-viral Type I IFN, like interferon-alpha (IFN-α), was inhibited in infected dendritic cells, and other host cytokines such as Interleukin (IL)-6, IL-10, TNF-α and IL-12 were therefore not induced [7]. At an initial time point of 3 hours, a transient increase in transcription expression level of genes which codes for pro-inflammatory cytokines like IL-1, IL-6 and IL-8 and then more extended release was fallowed at 24 h post infection of CSF virus [8]. Genes encoding coagulation factors, tissue factors, and vascular endothelial cell growth factor (VEGF), that are all essential in endothelial cell permeability, found their transcription levels also enhanced [8]. Many viruses have acquired the ability to infect immune cells, in addition to escaping the immune response

by modifying cell surface markers and cytokine induction, a mechanism that plays a significant role in coordinating the suppression of antiviral immune responses. An earlier study has shown that CSFV seems to have a specific affinity for immune system cells and can impede the immune response of a host. In CSF disease condition, both B- and T-lymphocytes are also reduced [9]. Before the advent of viremia, CD4+ and CD8+ T-lymphocyte levels fall significantly [10].

CSFV has a special affinity towards macrophage and monocytes, a lineage of phagocytic cells (reticulo-endothelial cells), that are primarily in the vascular endothelium[11]. These endothelial cells infection leads to an increase in vascular permeability, as well as lymphopenia, thrombocytopenia, coagulation disorders, and thymus and bone marrow degeneration [12, 13]. At the end stages of viral infection by CSFV, viral antigens may also carried out by Peripheral monocytes, lymphocytes, and granulocytes [14, 15]. Critical knowledge about pathological processes and the immune response to infection requires a specific study about the interactions between hosts and individual viruses. CSFV has a predisposition for immune system cells and may affect immune response gene expression. According to global transcriptional profiles of peripheral blood mononuclear cells following *in vivo* infection with CSFV, cellular genes showed a lower level of up- and down-regulation [16]. CD163 is a cell-surface glycoprotein molecule that is expressed only by phagocytic monocyte/macrophage cell populations and belongs to the family of scavenger receptor cysteine-rich domains (SRCR)[17]. The tightly regulated expression of CD163 by pro- and anti-inflammatory mediators suggests that it plays a critical role in the control of inflammatory processes [18]. In order to achieve a better overview of the concepts, we studied the expression of cellular genes like CD163 in response to CSFV infection in *in-vitro* module of macrophages using real-time RT-PCR linked with viral load in different breeds of pig like Landrace and native breed Ghurrah to get to know how CSFV impacts host gene expression linked with immune response.

Materials and Methods

Collection of blood. Isolation of PBMCs and culture of MDMs: The piglets were tested for Seronegativity for Maternally Derived Antibody and after that 10-12 ml of blood was collected aseptically from anterior vena cava of three landrace and three indigenous piglets, each in sterile heparin coated vacutainers from unvaccinated piglets for *in vitro* study. The blood samples were immediately transported from the farm to the laboratory on ice.

Isolation of PBMCs and culture of Monocyte Derived Macrophages: PBMCs were isolated using 3 ml of histopaque-1077 for each sample. First, blood was diluted with 1X PBS in 1:1 ratio and centrifuged at 3000 rpm for 30-35 min at room temperature (25°C) in swing out rotor centrifuge. Then whitish buffy coat was collected in fresh tube and treated with RBC lysis buffer till reddish tinge of RBCs get cleared. Final washing was given with 1X PBS and antibiotic, then another washing was given with RPMI-1640 complete medium. Supernatant was decanted and the pellet was suspended in 1 ml of RPMI complete growth medium (RPMI-1640 with antibiotic-antimycotic solution, Gentamicin and 15% Fetal calf serum). After that cell seeding was done in 6 well polystyrene BD Falcon plate at $0.6x10^6$ cells per well (2 ml for each well). The 2 plates separately taken for each Landrace and Ghurrah (Indigenous) cells and properly labelled. The cells were kept in CO_2 incubator for monocytes adherence at 37 ^{0}C and 5% CO_2 for 12-18 hours. After proper attachment of monocytes, discarded non-adherent cells and replaced with fresh RPMI 1640 complete media along with growth factor porcine GM-CSF (Sigma) @ conc. of 100 ng per ml. After 2-3 days of differentiation of monocytes in incubator, old media was replaced with fresh RPMI complete media and to facilitate the cell differentiation and proliferation, growth factor porcine-GM-CSF was added again at the rate of 100 ng per ml.

Infection of monocyte derived macrophages (MDMs) with CSF Virus

At 12-15 days of time monocytes adhered to plate were differentiated into phagocytic macrophages and the virulent strain of CSFV as a challenge virus was used for infection which was cultured in PK-15 (Porcine kidney) cell line at passage level 7 (P-7). Infected and passaged cells were frozen and thawed repeatedly in order to lyse the cells and release the virus in to the medium. Then contents were centrifuged and the virus was collected. The macrophages were infected on 15th day of culture by replacing 500 µL of media in the wells with $1x10^6$ $TCID_{50}$ at 1 M.O.I (multiplicity of infection). At 48 hpi the MDMs were harvested from the culture plates using TVG for trypsinization, 1X PBS for cell collection and Trizol for cell storage. After trypsinization cells were scraped with scraper and washed in 1X PBS and then stored in 200 µL of 1X PBS plus 800 µL Trizol solutions at 4 °C for overnight with proper labelling and then next day shifted to -80 °C till further use.

RNA extraction and cDNA synthesis: Total RNA was isolated from the harvested samples using Trizol reagent (Invitrogen) as protocol specified by the manufacturer, and then treated with DNase I for 15 min at 37 °C to eliminate contaminating DNA. The dried RNA pellets were resuspended in 30 µL of DEPC treated water. Total RNA quantity and purity were checked using spectrophotometry and electrophoresis respectively. The total RNA was either used directly for cDNA synthesis or stored at -80 °C until further use. According to the manufacturer's instructions, first-strand cDNA was synthesised using Oligo dT primers and Superscript II reverse transcriptase (Invitrogen). The synthesised cDNA was preserved at -80 °C for further use.

Quantitative real-time RT-PCR: IDT Primer quest tool software (Integrated DNA Technologies, Inc.) was used to design specific primers for CD163 receptor gene, 5'-UTR of CSFV, and the housekeeping gene GAPDH based on known swine sequences (Table 1). Real-time RT-PCR was performed on the Agilent Technologies AriaMX Real-Time PCR system. Reactions were performed in a 10 µL reaction mixture containing 5.0 µL of 2X SYBR green Master mix (Qiagen company), 2 µL of diluted cDNA or plasmid standard, 0.2 µL of each primer (10 µmol/µL), and 2.6 µL of nuclease free water (NFW). The thermal profile conditions included an initial heat-denaturing step at 95 °C for 2 min, 40 cycles of amplification at 95°C for 5 s, 58 °C for 30 s, and 72 °C for 30 s, followed by a melting curve analysis was performed to assess primer specificity and product quality by denaturation at 95°C for 30 s, annealing at 65 °C for 30 s, and finally melting at 95 °C for 30 s.

Table 1: Primer sequences used for real time RT-PCR in porcine MDMs.

Genes	Accession_ID (NCBI)	Primer sequence	Tm (˚C)	Amplicon length(bp)
CD163_SSC	EU016226.1	F: CAGGTTCTGGACGCATTT R: CACCAGCCTCATCTCTAAATC	58 ˚C	161 bp
GAPDH_SSC	NM_001206359.1	F: TCGGAGTGAACGGATTT R: CCGTGGGTGGAATCATA	58 ˚C	145 bp
5'UTR_SSC	MH487830.1	F:GGACAGTCGTCAGTAGTTCG R:CTGCAGCACCCTATCAGGTC	58 ˚C	151 bp

Results and Discussion

Classical swine fever (CSF) is a highly infectious and usually fatal swine disease caused by the classical swine fever virus (CSFV), a member of the pestivirus genus and the Flaviviridae family. CSFV is a sphere shaped virus particle with a diameter of 40-60 nm which is surrounded by a lipid enveloped nucleocapsid that consist of a positive-strand RNA genome of 12.3 kb [19]. CSFV leads to various forms of acute and chronic conditions in pigs by employing both virus virulence and host factors with the myeloid cell population, which includes the early targeted cells for infection, were monocytes, macrophages, and dendritic cells [20]. CSFV has devised a system to evade the antiviral response of these myeloid cells, permitting the virus to employ these migratory cells as transporters for delivery of pathogen to multiple tissues within the host, while likewise damaging immunocytes and somatocytes [21]. CD163 comes under a group of proteins with scavenger receptor cysteine-rich domains that are expressed only in monocytes and phagocytic macrophages. In pigs, CD163 has been demonstrated to have a role in the infection of monocytes/macrophages by various porcine viruses [22].

CD163 receptor is expressed exclusively in mature macrophages and there are various viruses reported to infect cells of monocyte/macrophage lineage [23-25]. In case of African swine fever virus it has been reported that virus permissiveness increases with maturation of macrophages and which was found correlated with high expression of CD163 receptor [22]. Similarly in our present study we found the relationship between CD163 receptor expression and Classical swine fever virus permissiveness or infection in porcine macrophages among two different breeds of swine which are Ghurrah and Landrace. Differentiated macrophages of these breeds were infected with CSFV *in-vitro* and cells were harvested at 48 hours post infection, from which RNA was extracted and cDNA prepared was used to analyze expression of CD163 receptor and also viral load by Real Time PCR. The cDNA was confirmed by conventional PCR and the product size of 161 bp (CD163) was found to be in agarose gel electrophoresis (Fig:1).

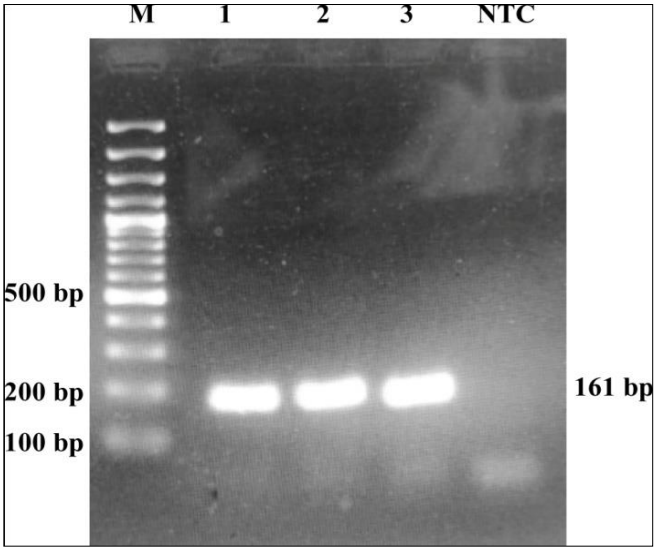

Fig 1: Agarose gel electrophoresis of CD163 receptor conventional PCR (M-100 bp marker, 1,2,3-Biological replicates, NTC-non template control).

Melt curve of CD163 receptor is depicted in Fig: 2. The Log_2FC of CD163 in Landrace was found to be 2.91±0.18 and in Ghurrah it was found to be 1.23±0.14 (Fig. 3). So, the expression of CD163 receptor was found to be high in Landrace as compared to Ghurrah breed.

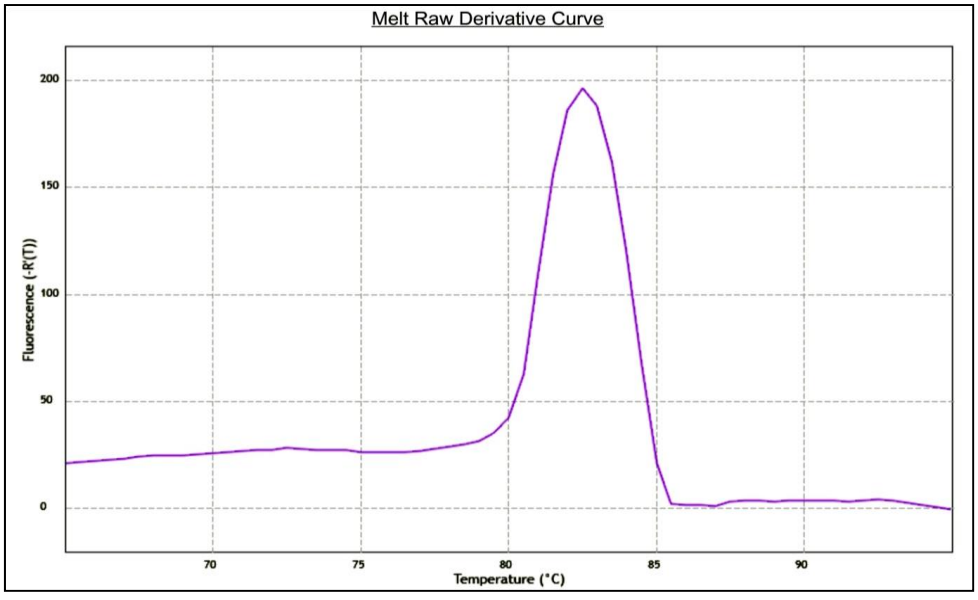

Fig 2: Melt curve of CD163 receptor in real time RT-PCR

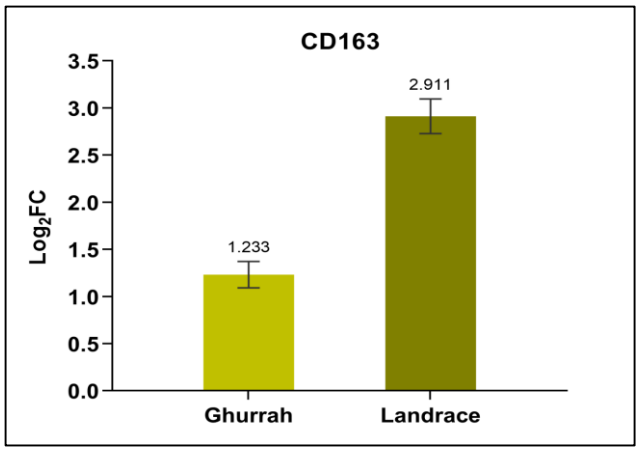

Fig 3: Bar graph depicting the expression of CD163 receptor in Ghurrah and Landrace breeds of Pig by real time RT-PCR (Landrace shows high expression compared to Ghurrah)

The amplification of standard curves for 5'-UTR region of CSFV was obtained using standard plasmids encoding the PCR products. The quantitative nature of the assay is demonstrated by the linear relationship between the log of the template copy number and the Ct value; a range with a correlation value (R) >0.99 is described. Melting curve analysis revealed that PCR amplified a single desired product. GAPDH was used as a housekeeping gene to correct for the amounts of total un degraded RNA in all samples. Expression of GAPDH was examined using real-time RT-PCR. Results showed that GAPDH mRNA levels did not vary between mock- and CSFV-infected cells, and the Ct parameter of GAPDH was found to vary between 19.24 and 19.95 (data not shown). The amplification efficiency of CSFV-specific cDNA was evaluated by comparing the standard curves. The standard curve for cDNA of the 5'-UTR of the CSFV genome was y= −3.266Log(x)+7.94, with a correlation coefficient R^2=0.991, and standard curve showed a 7-log dynamic range of amplification. The high correlation coefficient and large dynamic range indicate that the amplification efficiency is reproducible and therefore could be used to quantify CSFV-specific RNA in macrophages.

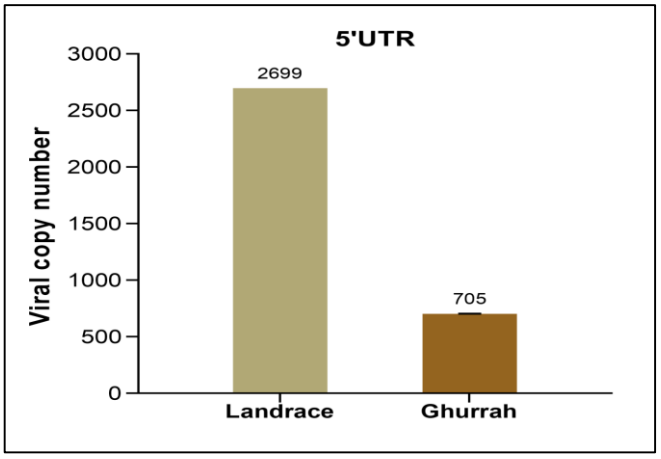

Fig 4: Bar graph showing the viral copy number of CSF virus in Landrace and Ghurrah breeds by absolute quantification of virus specific 5'-UTR region (Landrace shows high viral load compared to Ghurrah).

Viral load of CSFV in infected macrophages was identified by visualizing expression of 5'UTR region of CSFV genomic RNA by absolute quantification in Real Time PCR. Viral load or copy number of 5'UTR gene was found high in Landrace with respect to Ghurrah breed (Fig:4). So, in our study both CD163 receptor expression and viral load was found to be high in Landrace which signifies CSFV permissiveness increase in mature macrophages of Landrace more as compared to Ghurrah which was correlated highly with CD163 receptor expression. Therefore we determined by our study that CD163 receptor can serve as marker for CSFV infection in porcine macrophage infection. These overall findings can help to understand the pathogenesis of the disease, as well as in the designing of an effective vaccine for CSFV by identification of different chemical compounds capable of blocking host receptor by which virus interacts.

Conclusions

In short, our study concludes that classical swine fever virus infection in macrophages of two breed leads to increase in permissiveness of viral load vis a vis CD163 expression in Landrace compared to Ghurrah. So, it is first report for comparison of viral load and CD163 receptor expression in two breeds, further it may depicts to find out more potent vaccine in pig for CSFV.

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Conflict of Interest

The authors declare no conflict of interest. All the authors have read and approved the final version of the manuscript, and agree to be accountable for its contents.

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