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Expression of Markers of Innate Immune Response in Indigenous Pig of Northeast India in Comparison to Crossbred and Hampshire

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ABSTRACT

The function of innate immune system is to act as first line of defense against infectious/pathogenic challenge to the animals. A correlation between pathogen responses with genetic variation in immune response in pigs has been reported. Here we present the study on expression of innate immune response markers (IL-6, IL-12, INF-γ, IL-1β, TNF-α and c - reactive protein) in indigenous pig along with crossbred and Hampshire. The results suggest there exists difference in the expression pattern of markers in various breeds. This information may serve as an indicator for rational design or evaluation of immune response of porcine.

1. Introduction

The major immune cells are macrophages, neutrophils and natural killer cells which recognizes pathogen-associated molecular patterns (PAMPs) via specific host pattern recognition receptors (PPRs) (Kelly and King 2001). The recognition of PAMPs causes the activation of the compliment system and trigger pro-inflammatory and antimicrobial responses (Akira and Takeda, 2004). PRR-induced signal transduction pathways ultimately result in the activation of gene expression and synthesis of a broad range of molecules, including chemokines, cell adhesion molecules, immune-receptors and cytokines such as the interleukins (IL-1, IL-6), interferon-gamma (INF-γ), tumor necrosis factor (TNF - α) (Kelly and King, 2001; Akira *et al.,* 2006), together they orchestrate the early host response to infection and at the same time direct the adaptive immune response. PRRs also recognize host factors as "danger" signals, when they are present in aberrant locations or abnormal molecular complexes as a consequence of infection, inflammation, or other types of cellular stress (Matzinger, 2002). C-reactive protein is an acute phase serum protein and a mediator of innate and adaptive immunity (Chomdej *et al.*, 2004). Its estimation can be used as a tool in animal health and welfare monitoring (Gutierrez *et al.,* 2009).

Sudden and substantial changes in the environment cause stress to the organism to mount specific cytoprotective responses for survival, including those at the cellular level (Fulda *et al.,* 2010). Incidence in disease classifications and distributions of leukocytes in serum of pigs are low to moderately heritable (Henryon *et al.*, 2001; 2002). Genetic variation in response to modulation of the immune system also has been demonstrated (Edfors - Lilja *et al.*, 1995; Mallard *et al.*, 1998; Wilkie and Mallard, 1999). This paper reports the differential expression of genes responsible for innate immune response of the different breeds of pig - the Indigenous, Crossbred and Hampshire.

2. Materials and Methods

Peripheral blood mononuclear cell (PBMC) isolation and stimulation

Blood was collected aseptically from three different breed of pigs namely Indigenous, Crossbred (50%) and Hampshire (6 no from each breed) in anticoagulant (heparin@ 40-60 IU) containing syringes. Approximately 3 ml of whole anti-coagulated blood was mixed with equal volume of sterile phosphate buffer saline (PBS). 6 ml of Ficoll-Paque 1077 (Sigma) was pipetted into a 15 ml centrifuge tube. Equal volume of diluted blood was gently layered onto the Ficoll.

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The sample was centrifuge at 18,000 g for 45 min at room temperature. The PBMC layer at diluted plasma/Ficoll interface was collected in fresh tubes and washed with sterile PBS thrice (Buchanan *et al.*, 2011). The cells were counted and dispensed in 96 well round bottom plate (Corning, USA). 1 x 10^5 cells/100 µl were seeded in each well using RPMI medium supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich). Cells in triplicate wells were stimulated with 100µl of Concavalin A (Sigma-Aldrich) at a final concentration of 2µg/ml, control cells were added with media alone and incubated at 37°C in an incubator with 5% CO_2 for 24 hours. Cells were harvested and stored at –20 °C until they are processed for gene expression analysis.

Isolation of total RNA and expression of β actin

Total RNA was extracted from PBMCs using the TRIzol reagent (In-vitrogen) as described by Duan *et al.* (2014). Reverse transcription was performed as per the manufacturer's instruction using the Transcriptase Kit (Takara). The relative expression levels of the target genes were determined by quantitative real time PCR. Beta-actin was used as a housekeeping gene/internal control to normalize the expression of target genes. Each 20 µl PCR reaction in PCR fast reaction tubes (Applied Biosystems), contained 7.5 µl of NF water, 10 µl of SYBR Green I Universal Master Mix (Applied Biosystems), 0.5 µl (10 picomole concentration) of each forward and reverse primer (IDT, USA) and 2 µl of cDNA. In an Applied Biosystems 7900 real-time PCR machine (ABI Biotechnology), the PCR cycles used were: 1 cycle of 95 °C for 3 min to activate the enzyme, followed by 40 cycles of 95 °C for 1 second (denaturation) and 60 °C for 60 seconds (annealing and extension). A non template control was also used in each run to access specificity of primers and possible contamination.

Expression of immune response genes

Published primers sequence for the selected genes (IL-6, IL-12, INF-γ, IL-1β, TNF-α and c - reactive protein) are used as presented in table 1 (Supplementary page). The relative quantification of gene amplification by RT-PCR was performed using the value of the threshold cycle (Ct). The comparative Ct value method (Livak and Schmittgen, 2001) was employed to quantify the expression levels of the selected genes relative to those of β-actin using the following formula: $\Delta\Delta C_T = (C_T \text{ target gene} - C_T \text{ β-actin})_{\text{treated}} - (C_T \text{ target gene} - C_T \text{ β-actin})_{\text{calibrator}}$. Using the $2^{-\Delta\Delta C}{}_T$ method, the data are presented as the fold change in gene expression normalized to an endogenous control (β-actin) and relative to the untreated control/calibrator (C).

Statistical analysis

All the data are expressed as mean with their standard errors. Statistical analyses were carried out using Graph Pad Prism 6 software (Graph Pad Software, Inc., San Diego, California, USA) using one-way ANNOVA followed by the Turkey's multiple comparison test. Significance was set at *P<0.05.*

Results

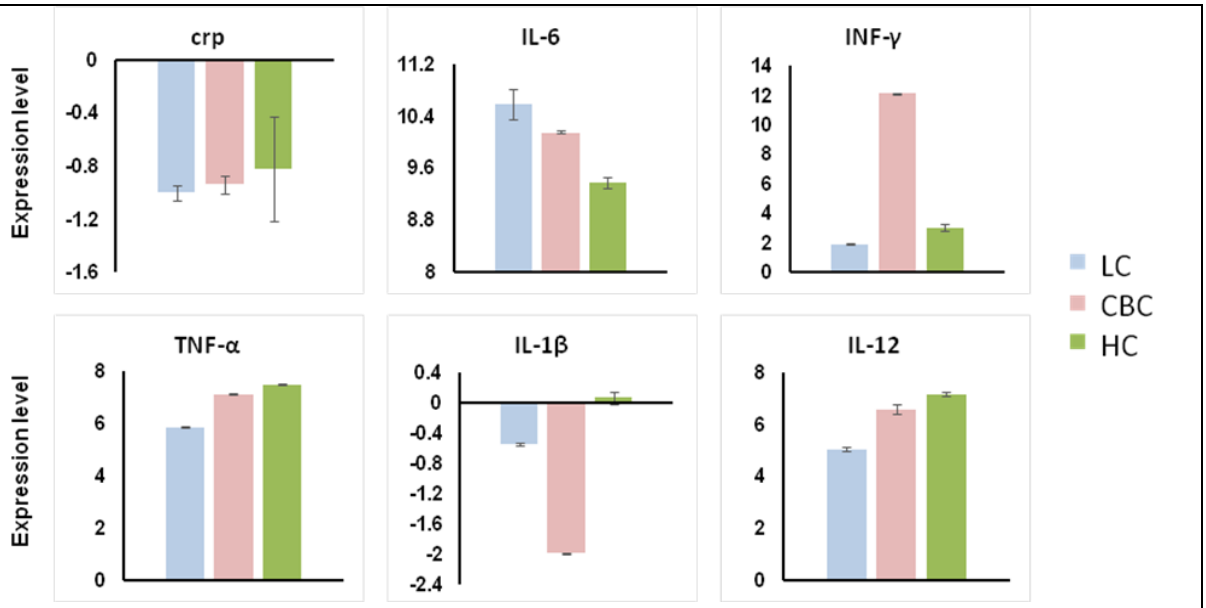

Figure 1A. The constitutive expression level of the target gene in various breeds (LC, CBC, HC) – crp, IL-6, INF-γ, TNF-α, IL-1β, IL-12 normalized to an endogenous control (β-actin), P value was set at *P<0.05.*

The expression of immune markers; c-reactive proteins (crp) and cytokines - IL-6, IL-12, IL-1β, INF-γ and TNF-α are as shown in figure 1 a & b. There is a high variation in the expression of cytokines in the different breeds of pigs *i.e.* Local (LC), Cross-bred (CBC) and Hampshire (HC). The constitutive expression of IL-6 in Khasi local (LC) was observed to be higher than Cross-bred (CBC) and Hampshire (HC). INF-γ expression in CBC is significantly higher than LC and HC ($P<0.05$). The crp, TNF-α, IL-1β and IL-12 expression was higher in HC relative to CBC and LC (Fig 1A). From the fold change calculation ($2^{-\Delta\Delta C_T}$) of gene expression, stimulation with mitogen (concanavalin A) did not cause any significant change in the expression of crp in all the three breed of pigs relative to the respective un-stimulated controls while a decrease in IL-6 genes in LT was observed. An increase in expression of IFN-γ in CBT and HT, an overall decrease of TNF-α expression in all the three breed, a decrease in expression of IL-1β in CBT and HT and a decrease of IL-12 in CBT only relative to their un-stimulated control respectively was observed (Fig 1B).

Discussions

The indigenous pig of Meghalaya also known as the Megha Sniang (registered breed with NBAGR, Karnal) has a small body size at maturity with small litter size. However, they are better adapted to local climatic conditions in terms of health and disease resistance. Hampshire pigs are muscular, rapid growers and exhibit good carcass quality as meat animals. Cross-bred was developed by the division of Livestock Production, ICAR-NEH, Umiam, Meghalaya, India, by crossing the local pigs of Meghalaya with Hampshire. Cross-bred performed better than the local breed in terms of body weight gain, litter size and weight at birth and weaning, feed conversion efficiency, carcass quality for meat, *etc*. Therefore, the expression of various markers responsible for innate immune response were analysed along with expression of C-reactive protein in mononuclear cells of these three different breeds of pigs. The findings showed the constitutive expression of the different immune markers - IL-1β, which is an important mediator of inflammatory response (Ren and Torres, 2009) is higher in Hampshire along with TNF-α, an important cell signalling molecule responsible for systemic inflammation (Diez-Pina, 2009) and IL-12, responsible for bridging the innate and adaptive responses (Trinchieri, 1995), the difference in expression of IL-6, an important pro- and anti-inflammatory cytokine (Scheller, 2011) was non-significant and IFN-γ, which functions as an immediate immune responder against infectious microorganism (D'Elios *et al.*, 2011) was higher in Hampshire. The difference in the expression of crp was non-significant (Fig. 1A). Stimulation with mitogen (Con A) causes a down-regulation of TNF-α in all the three breeds of pigs, IL-1β in Cross-bred and Hampshire pigs and IL-12 in Cross-bred pigs, whereas an up-regulation in INF-γ in Cross-bred and Hampshire.

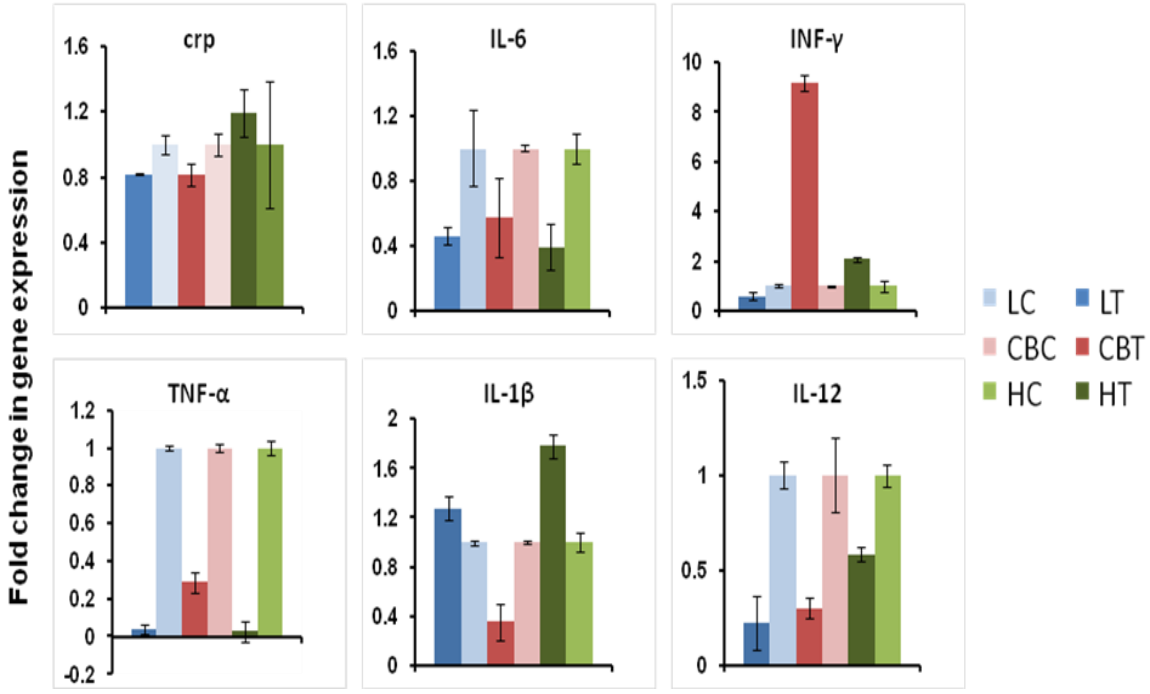

Figure 1B. The fold change in expression of the target gene in treated (LT, CBT, HT) group – crp, IL-6, INF-γ, TNF-α, IL-1β, IL-12 normalized to an endogenous control (β-actin) and relative to the respective untreated control/calibrator (LC, CBC, HC), P value was set at *P<0.05.*

The down regulation of TNF- α expression upon stimulation may be due to the IL-6. A similar observation was reported by Schindler *et al.* (1990) in humans upon stimulation with PHA mitogen. The increased in IL-6, IL-10, and IFN-γ mRNA expression in porcine reproductive and respiratory syndrome virus-infected pigs was reported by Feng *et al.* (2003). The constitutive mRNA expression of IL-12, IL-6 and TNF- α in piglet is stronger and the expression of IL-12 and IL-6 mRNA may reflect an inflammatory reaction against the exo- and endogenous foreign bodies occurring in the lymphatic organs, especially in the tonsil (Mikami *et al.*, 2002). The plasma concentration of C- reactive protein has been used clinically in pigs to monitor inflammation and infection (Kuji *et al.*, 2007) with increases of up to 38-fold reported during acute inflammation (Parra *et al.*, 2006). C-reactive protein expression in all the three breed of pigs did not show any change in expression levels when stimulated.

The role of cytokines need to be investigated further to see if any relation to environmental stress is responsible for its constitutive up-regulation/down-regulation as the temperature and humidity bearing on the animal and health status is also to be considered. The differential expressions of immune response genes may be due to regulation by cell stress proteins which may dictate the inflammatory profile of the immune response during infection and disease (Muralidharan and Mandrekar, 2013) since different proteins which responds to cellular stress caused by various environmental insults interact with and regulate signalling intermediates involved in the activation of innate and adaptive immune responses.

Conclusion

The local pig suited to the climatic conditions of the region, though small in size, reach maturity at an early age and prolific litter size have very few data available on production performance or disease resistance. The expression of immune markers in local pig varies with crossbred and exotic pig suggesting there exist differences in natural response against infections. With this study, rational design or evaluation of immune response of porcine to other pathogens/various climatic stress conditions can be developed and further evaluated.

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References

Akira S., K Takeda (2004). Toll-like receptor signalling. Nature Reviews. *Immunology* 4: 499–511.

Akira S., Uematsu S, Takeuchi O (2006). Pathogen recognition and innate immunity. *Cell* 124: 783–801.

Buchanan R.M., Popowych Y, Arsic N, Townsend H.G, Mutwiri G.K, Potter A.A, Babiuk LA, Griebel P.J, Wilson HL (2011). B-cell activating factor (BAFF) promotes CpG ODN-induced B cell activation and proliferation. *Cellular Immunology* 271: 16-28.

Chomdej S., Ponsuksili S, Schellander K, Wimmers K (2004). Detection of SNPs and linkage and radiation hybrid mapping of the porcine C-reactive protein (CRP) gene. *Animal Genetics* 35: 469–470.

D' Elios M.M., Benagiano M, Bella C.D, Amedei A (2011). T-cell response to bacterial agents. *Journal of Infection in Developing Countries* 5(9): 640-645.

Diez-Pina J.M., Fernandez-Aceñero M.J, Llorente-Alonso M.J, Diaz-Lobato S, Mayoralas Sagrario, Florez A (2009). Tumor necrosis factor alpha as a marker of systemic and local inflammation in "healthy" smokers. *International Journal of General Medicine* 2: 9-14.

Duan F., Duitama J, A.l Seesi S, Ayres C.M, Corcelli S.A, Pawashe A.P, Blanchard T, Mcmahon D, Sidney J, Sette A, Baker B.M, Mandoiu I.I, Srivastava P.K (2014). Genomic and bioinformatic profiling of mutational neoepitopes reveals new rules to predict anticancer immunogenicity. *Journal of Experimental Medicine* 211(11): 2231-48.

Edfors-Lilja I., Gustafsson U, Duval-Ilflah Y, Andersson L.A (1995). The porcine intestinal receptor for Escherichia coli K88ab, K88ac: Regional localization on chromosome 13 and influence of IgG response to the K88 antigen. *Animal Genetics* 26: 237–242.

Feng W.H., Tompkins M.B, Xu J.S, Zhang H.X, McCaw M.B (2003). Analysis of constitutive cytokine expression by pigs infected in-utero with porcine reproductive and respiratory syndrome virus. *Veterinary Immunology and Immunopathology* 94(1-2): 35-45.

Fulda S., Gorman A.M, Hori O, Samali A (2010). Cellular stress responses: cell survival and cell death. *International Journal of Cell Biology* 2010: 214074, 1-24.

Henryon M., Juul-Madsen H.R, Berg P (2002). Genetic variation for total and differential numbers of leukocytes in growing pigs. In: Proceedings of the 7th World Congress on Genetics Applied to Livestock Production, Montpellier, France, 13: 1-4.

Kelly D., King T.P (2001). A review- Luminal bacteria and regulation of gut function and immunity. In: Manipulating Pig Production VIII (Ed. P D Cranwell)

Kuji T., Masaka T, Li L, Cheung A.K (2007). Expression of C-reactive protein in myointimal hyperplasia in a porcine arteriovenous graft model. *Nephrology Dialysis Transplantation* 22: 2469–2475.

Livak K.J., Schmittgen T.D (2001). Analysis of relative gene expression data using realtime quantitative PCR and the 2-^^CT method. *Methods* 25: 402-408.

Mallard B.A., Wilkie B.N, Kennedy B.W, Gibson J, Quinton M (1998). Immune responsiveness in swine: Eight generations of selection for high and low immune response in Yorkshire pigs. In: Proceedings of the 6th World Congress on Genetics Applied to Livestock Production, Armidale, NSW, Australia, 27: 295-302.

Matzinger P., (2002). The danger model: a renewed sense of self. *Science* 296: 301–305

Mikami O., Muneta Y, Mori Y, Yokomizo Y, Nakajima Y (2002). Expression of proinflammatory cytokine mRNA in the lymphatic organs of adult and neonatal pigs. *Veterinary Immunology and Immunopathology* 90(3-4): 203-7.

Muralidharan S., Mandrekar P (2013). Cellular stress response and innate immune signaling: integrating pathways in host defense and inflammation. *Journal of Leukocyte Biology* 94(6): 1167-1184.

Parra M.D., Fuentes P, Tecles F, Martınez-Subiela S, Martınez JS, Munoz A, Ceron JJ (2006). Porcine acute phase protein concentration in different diseases in field conditions. *Journal of Veterinary Medicine* B 53: 488–493.

Ren K., Torres R (2009). Role of interleukin-1β during pain and inflammation. *Brain Research Reviews* 60(1): 57-64.

Schindler R., Mancilla J, Endres S, Ghorbani R, Clark S.C, Dinarello C.A (1990). Correlations and Interactions in the Production of Interleukin-6 (IL-6), IL-1, and Tumor Necrosis Factor (TNF) in Human Blood Mononuclear Cells: IL-6 Suppresses IL-1 and TNF. *Blood* 75(1): 40-47.

Trinchieri G., (1995). Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annual Review of Immunology* 13: 251-76.

Wilkie B., Mallard BA (1999). Selection for high immune response: An alternative approach to animal health maintenance. *Veterinary Immunology and Immunopathology* 72: 231–235.

Scheller J., Chalaris A, Schmidt-Arras D, Rose-John S (2011). The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochemica et Biophysica Acta* 1813: 878–888.

Henryon M., Berg P, Jensen J, Anderson S (2001). Genetic variation for resistance to clinical and subclinical diseases exists in growing pigs. *Animal Science* 73: 375–387.

A.M., Martınez-Subiela S, Eckersall P.D, Ceron J.J (2009). C-reactive protein quantification in porcine saliva: A minimally invasive test for pig health monitoring. *Veterinary Journal* 181: 261–265.