FUNCTIONAL GENOMIC ASSESSMENT OF PORCINE DISEASE RESISTANCE : REAL TIME ASSAYS OF PORCINE IMMUNE GENE EXPRESSION

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INTRODUCTION

Identification of the exact genes involved in regulating disease resistance in swine is essential for our goal of identifying pigs which are genetically superior in their ability to naturally resist infections. Different authors have attempted to design methods to select more disease resistant pigs (Wilkie and Mallard, 1999 ; MacKenzie and Bishop, 2001). More tools to verify immune genes regulating disease resistance in pigs will be essential to expand these approaches and to verify data that will be derived from microarray experiments. Use of functional genomic tools to assess gene expression during diseases will expand our understanding of genes controlling disease responses, as is currently underway with humans (Boldrick *et al*., 2002).

Therefore we are developing real-time PCR for assessing mRNA levels of immune regulatory molecules, particularly the cytokines and their receptors. These assays will be used to compare immune phenotypes of pigs, to determine their immune cytokine responses at different developmental stages and to assess their responses to vaccination and deliberate infections.

MATERIALS AND METHODS

Cell and mRNA and cDNA preparations. Pig blood samples were collected in vacutainer tubes containing EDTA and peripheral blood mononuclear cells (PBMC) using Ficoll gradients as described previously (Solano-Aguilar *et al*., 2000). PBMC ($5.0x10^7$ cells) were cultured at 37°C either with phorbol myristate acetate (PMA 5 ng/ml) with Concanavalin A (ConA 2.5µg/ml) or with Phytohemaglutinin (PHA 10 µg/ml) with interleukin-2 (rHuIL-2 50 U/ml. Cells were harvested at 0, 2, 4, 20 and 44 hours, pelleted and frozen at –70 °C until RNA extraction was performed using RNeasy (Qiagen). Reverse Transcription (Improm-II Reverse Transcription, Stratagene) was performed using 1 µg RNA per 20 µl RT reaction.

Cytokine mRNA assay. Real Time PCR was performed on an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems) and results analyzed using Sequence Detection System software version 1.7 (PE Applied Biosystems). Primers were designed using Primer Express software v.1.5a (PE Applied Biosystems). Forward and reverse primers were optimized and used at 50-900 nM with probes at 150-200 nM with the housekeeping 18S rRNA Control (PE Applied Biosystems). Reporter dye : VIC (HPRT and r18S) / FAM (all cytokines probes) with TAMRA quencher dye and ROX reference dye. The PCR reaction was performed in 20 µl on 10 ng cDNA (RNA equivalent) using Brilliant Quantitative PCR Core

Reagent kit (Stratagene) and 5.5 mM $MgCl_2$, 1X buffer, 200 µM dNTP, 1U SureStart Taq DNA polymerase starting at 50 °C, 2 min ; 95 °C, 10 min, followed by 45 cycles [95 °C, 15 sec ; 60°C, 1 min]. In every plate, for each set of primers and probes, no template (NTC) and no amplification controls (NAC) were run.

Quantitation of Gene Expression. For relative quantitation analysis the amplification of a housekeeping gene (rRNA or HPRT) is used to standardize the amount of cDNA in each reaction. Target gene (cytokine or receptor) amount is calculated by normalizing it to the reference gene value. The cycle number at which the fluorescent signal reaches the threshold value (C_T) is used for quantitation analysis. Threshold line is obtained by using the 3 to 15 cycle baseline values ± 10X the standard deviation. The standard curve for each gene is prepared using serial dilutions of stock cDNA (Anonymous 1998) ; using the log cDNA (x), average C_T (y) and slope (m), the linear regression is calculated [y = mx + b]. Using the Comparative C_T Method the amplification efficiencies of the target (cytokine) and reference (HPRT, r18S) genes are used to determine the ΔC_T (target C_T – reference C_T) and related to a calibrator ($\Delta\Delta C_T = \Delta C_T$ – Calibrator ΔC_T). The expression of Interferon-□ in cultured PBMC determined by using the formula: $2^{-\Delta\Delta CT}$ using the target gene IFN-γ, reference HPRT, and calibrator = 0 hour culture.

RESULTS AND DISCUSSION

Real-time PCR has advantages over other mRNA assays currently in use in that they are semi-quantitative, accurate but time and labor intensive. Thus quantitative real-time TaqMan PCR assays were developed for a panel of porcine cytokines and their receptors. To date assays have been validated for cytokines including IFN-γ, IFN-α, TNF-α, IL-2, IL-4, IL-5, IL-10, IL-12 p35 and p40, IL-13, IL-15, IL-18, and the housekeeping genes 18srRNA and HPRT. In addition assays were developed for cytokine receptors: IL-4R, IL-5R, IL-12Rβ2, and IL-13R.

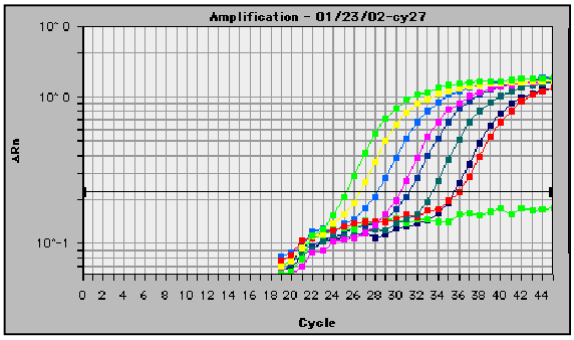

Figure 1. Establishment of the real-time PCR assay for IFN-γ. Increasing dilutions of cDNA were assessed for mRNA. The black line indicates the background ; the different colored lines the results for each amount of cDNA. C_T values for mRNA quantitation were calculated and plotted in Figure 2.

Primers and fluorescent probes were designed across adjacent exons when possible. RNA was extracted from PBMCs with or without activation, reverse transcribed and cytokine message assayed. For each cytokine the assay was optimized for primer and probe concentrations (figure 1). The assays were validated by 1) use of specific primer and probe sequences, 2) comparison of results with semiquantitative RT-PCR, and 3) expression in tissues from pigs infected with the parasites, *Toxoplasma gondii* or *Ascaris suum*.

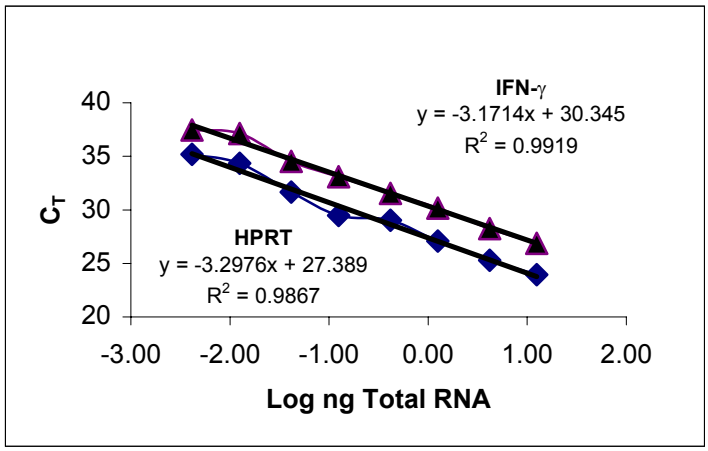

Figure 2. Establishment of Linearity of the IFN-γ and HPRT assays. C_T values for cDNA quantitation were obtained from the assay as noted in Figure 1 and calculations performed as stated in Materials and Methods.

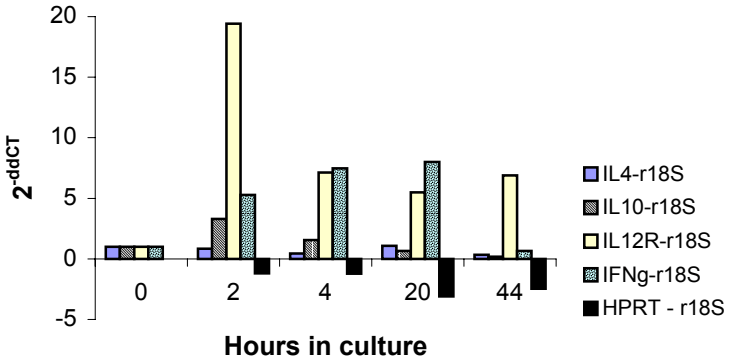

Figure 3. Relative quantitation of mRNA for IL-4, IL-10, IL12Rβ2 and IFN-γ expression. Pig PBMC were cultured for 2, 4, 20 and 44 hours with 10ug PHA and 50U HuIL-2/ml. Relative mRNA levels were calculated by the comparative C_T method using 18SrRNA as reference.

Figure 2 gives an example of the assay for the cytokine mRNA for IFN-γ and the housekeeping gene, HPRT, expression in PHA/IL-2 stimulated PBMC using increasing amounts of cDNA to standardize the assay. Figure 3 shows the relative differences in gene expression for IL-4, IL-10, IL-12Rβ1, IFN-γ and HPRT from cells cultured over 44 hours with mitogen and IL-2.

These assays will be used to compare immune phenotypes of lines of pigs to assess gene expression at different developmental stages, or after vaccinations or infectious disease challenge. They will also be useful diagnostic tools to differentiate immune type 1, type 2, and regulatory T cell subset activities in swine during development, and following infections or vaccinations. They will help producers select swine with improved genetic responses.

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