เชียงใหม่สัตวแพทยสาร 2551;6(2):177-183.

Original article

AN Interleukin-10 and interleukin-18 gene expressions in porcine monocytes in response to mitogens

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Abstract Most studies on porcine cytokine gene expression are conducted in peripheral blood mononuclear cells and T cells. Such studies in monocytes are little. In monocytes, porcine cytokine gene expression usually uses lipopolysaccharide (LPS) as stimulus.The use of lectin group of mitogens ie. concanavalin A (conA), phytohemagglutinin (PHA), and pokeweed mitogen (PWM) is limited. The objective of this study is to evaluate and compare the efficiency of conA, PHA, PWM, and LPS in inducing interleukin-10 (IL-10) and IL-18 gene expression in porcine monocytes. Results show that conA, PHA, PWM, and LPS are capable of inducing IL-10 gene expression when used at 5 and 10 µg/ml and incubated with monocytes for 12 hours, and at 1, 5, and 10 µg/ml and incubated with monocytes for 24 hours. PHA, PWM, and LPS are significantly more potent in inducing IL-10 gene expression than conA. PWM and LPS are capable of inducing IL-18 gene expression when used at 5 and 10 µg/ml and incubated with monocytes for 24 hours. Their IL-18-inducing efficiency is not significantly different from each other.This study suggests that certain lectin group of mitogens and LPS can be used as stimuli for positive control of IL-10 and IL-18 gene expression study in porcine monocytes. **Chiang Mai Veterinary Journal 2008;6(2):177-183.**

Keyword: interleukin-10, interleukin-18, porcine, monocyte, mitogen

Introduction

Extensive studies on porcine cytokine gene expression have been conducted in peripheral blood mononuclear cells (PBMC) and T cells. Such studies in monocytes are little. Porcine PBMC and T cells express cytokines in response to various stimuli eg. viruses, bacteria, oligodeoxynucleotides, and lectin group of mitogens.[1-5] Of these, lectin group of mitogens ie. concanavalin A (conA), phytohemagglutinin (PHA), and pokeweed mitogen (PWM) is most potent stimulator. It is often used as stimulus for positive control of porcine cytokine gene expression studies.[1, 2, 6, 7] Porcine PBMC and T cells express cytokine with varying degrees of expression in response to different lectins. For examples, the cells express interferon gamma (IFNγ) gene more vigorously in response to conA and PWM than PHA, while they express interleukin-4 (IL-4) gene more strongly in response to PHA than conA and PWM.[1] In porcine monocytes, stimuli used to induce cytokine gene expression are of bacterial components eg. lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan, and plasmid DNA.[8-13] The use of lectin group of mitogens to stimulate porcine monocytes is limited.[10, 13] This study aims to evaluate and compare the efficiency of conA, PHA, PWM, and LPS in inducing IL-10 and IL-18 gene expression in porcine monocytes. The expression of IL-10 gene by porcine monocytes has been reported only after LPS stimulation, and the expression of IL-18 gene by porcine monocytes has not been studied.[8] Results reveal that PHA, PWM, and LPS have comparable efficiency in inducing IL-10 gene expression; and PWM and LPS have comparable efficiency in inducing IL-18 gene expression. This study suggests potential use of certain lectins and LPS for stimulation of IL-10 and IL-18 gene expression in porcine monocytes.

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Materials and methods

1. Mitogens

ConA and PWM were purchased from Sigma (St. Louis,MO). PHA was purchased from Biochrom Ag (Germany). LPS was purchased from Fluka (Germany)

2. Isolation of PBMC

Blood were collected from four 24-week-old pigs that were seronegative for porcine reproductive and respiratory syndrome virus. The blood were placed in 0.1 volume of 0.5M ethylene diamine tetra-acetic acid (EDTA) solution (J.T.Baker, Phillipsburg, NJ), then diluted with equal volume of phosphate buffered saline (PBS) and layered onto lymphocyte separation medium (Histopaque®-1077, Sigma, St. Louis, MO), and centrifuged at 1,200xg and 25°C for 30 minutes. PBMC were collected, washed once with PBS, and centrifuged at 270xg and 4°C for 10 minutes. Contaminating red blood cells were lysed with cold buffered water (0.156M ammonium chloride, 10mM sodium bicarbonate, and 1mM EDTA) for 5 minutes. PBMC were resuspended in $RPMI^{++}$ (RPMI-1640 with L-glutamine, 10% heat-inactivated fetal bovine serum, and 1% tissue culture penicillin (10,000IU) /streptomycin (10,000µg/ml) /amphotericin B (25 µg/ml) (Gibco, Grand Island, NY)) and counted by hemocytometer (Bright-Line®, Hausser Scientific, Horsham, PA). PBMC concentrations were adjusted to $5x10^6$ cells/ml in $RPMI^{++}$.

3. Cell cultures

Two hundreds µl of PBMC in $RPMI^{++}$ ($5x10^6$ cells/ml) were placed into each well of a 96-well flat-bottom plate (Nunc, Denmark) and incubated for 12 hours at 37°C in a humidified, 5% CO_2 atmosphere. Non-adherent cells were removed and adherent cells were gently washed once with 150 µl pre-warmed (37°C) $RPMI^{++}$ to obtain monocytes.[14] Adherent monocytes then received 200 µl of $RPMI^{++}$ and 50 µl of conA, PWM, PHA, or LPS (prepared in $RPMI^{++}$). The final concentration of each mitogen was either 1, 5, or 10 µg/ml. Negative control of the experiment was monocytes receiving only $RPMI^{++}$ (250 µl). Cells were cultured at 37°C in a humidified, 5% CO_2 atmosphere for 12 and 24 hours after receiving mitogens.

4. Determination of cell viability

The viability of monocytes prior to and after stimulation with mitogens for 12 and 24 hours was evaluated by trypan blue dye exclusion assay. In each well, culture media were removed and replaced with 150 µl ice-cold PBS plus 5mM EDTA. Culture plates were incubated on ice for 10 minutes. Ten µl of harvested monocyte suspension were incubated with equal volume of 0.4% trypan blue (Gibco, Grand Island, NY) at room temperature for 5 minutes. Half of the resulting mixtures were placed in a hemocytometer and determined for cell viability.

5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

5.1 RNA extraction

At the end of 12- and 24-hour stimulation period, monocytes were harvested as described above and placed into sterile microcentrifuge tubes (Bioline, Taunton, MA). Cells were pelleted at 270xg and 4°C for 1 minute, washed once with ice-cold PBS, resuspended with 200 µl RNAlater (Sigma, St. Louis, MO), and stored at -20°C until RNA extraction. Total RNA was extracted, using the RNeasy® mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Contaminating DNA was eliminated by Dnase I (Fermentas, Glen Burnie, MD). Total RNA was eluted in 40 µl of RNase-free water.

5.2 One-step RT-PCR

One-step RT-PCR was performed using SuperScript™ III One-Step RT-PCR System with Platinum® Taq kit (Invitrogen, Carlsbad, CA). The reaction was carried out in a total volume of 50 µl, consisting of 5 µl total RNA template, 2 µl primer mix (1 µl each of forward and reverse primer (Table 1)), 40 units RNase inhibitor (RiboLock™, Fermentas, Glen Burnie, MD), and RT-PCR buffer and reverse transcriptase/Taq polymerase mix provided with the kit. The RT-PCR condition was cDNA synthesis at 55°C for 30minutes; denaturation at 94°C for 2 minutes; 33 cycles of 'denaturation' at 94°C for 30s, 'annealing' at 55°C for 45s, and 'extension' at 68°C for 1 minute; and final extension at 72°C for 5 minutes. The number of PCR cycle was optimized by performing different numbers of PCR cycle and selecting the cycle that none of the PCR products reached the plateau phase during PCR amplification (data not shown).

5.3 Quantification of the PCR products

Gel electrophoresis of PCR products was performed on 2.5% agarose (Research Organics, Cleveland,OH) in Tris-borate-EDTA buffer (National diagnostics, Atlanta, GA) with 0.5 µg/ml ethidium

Table 1. Primer sequence designed for one-step RT-PCR

Gene	Primer sequence	Accession #	Final concentration (nM)	Product (bp)
IL-10	5'TCAGCACTGCTCTATTGCCTG3' (forward)	L20001	200	472
	5'TGAAGATGTCAAACTCACCCA3' (reverse)		200	
IL-18	5'TGGAATCGGATTACTTTGGCA3' (forward)	AB010003	200	347
	5'CTTATCATCATGTCCAGGAACAC3' (reverse)		200	
GAPDH	5'AGGACGTGCAGAAGAGCAGAGCGA3' (forward)	AF017079	50	578
	5'GCATTGCTGATGATCTTGAGGCTG3' (reverse)		50	

bromide (Bio Basic Inc., Canada). Images of PCR products on agarose gel were visualized by ultraviolet illuminator, and captured and analyzed for density with Quantity One software (version 4.5.0, Bio-Rad, Hercules, CA). The expression of IL-10 and IL-18 genes were presented as percentage ratio of cytokine: glyceraldehydes-3-phosphate dehydrogenase (GAPDH) expression of the same animal.

6. Statistical analysis

All statistical analyses were performed using the JMP6 software (SAS Institute Inc., Cary, NC). Mean differences of %viability of monocytes, %IL-10, and %IL-18 gene expression were tested among types of mitogen at the same concentration and stimulation period, using one-way analysis of variance (ANOVA), followed by Dunnett's test using mean of negative control as a control group. The highest mean %IL-10 and %IL-18 of each mitogen was compared for mean difference, using one-way ANOVA, followed by Tukey–Kramer honestly significant difference test. $P<0.05$ was set as statistically significant level

Results

1. Effect of mitogens on monocyte viability

The viability of monocytes prior to stimulation with mitogens was 80-90% in all four animals. The percentage of cell viability was slightly reduced to 75-85% and 70-80% after 12 and 24 hours of cell stimulation with mitogens, respectively. No significant effects of type and concentration of mitogens and stimulation period were detected on monocyte viability (data not shown).

2. IL-10 gene expression

Significantly increased IL-10 mRNA expression of monocytes was detected in response to conA, PHA, PWM, and LPS after 12 and 24 hours of stimulation. At 12 hours of stimulation, significantly increased IL-10 mRNA expression was detected in response to PHA and LPS at 5 µg/ml, and to all mitogens at 10 µg/ml (Fig. 1). At 24 hours of stimulation, significantly increased IL-10 mRNA expression was detected in response to PHA, PWM, and LPS at 1 µg/ml, and to all mitogens at 5 and 10 µg/ml (Fig. 2). There was no significant difference in efficiency of mitogens in inducing IL-10 mRNA expression at 12 hours of stimulation (Fig. 1). However, at 24 hours of stimulation, PHA, PWM, and LPS were significantly more efficient than conA in inducing IL-10 mRNA expression (Fig. 2).

3. IL-18 gene expression

No significantly increased IL-18 mRNA expression was detected in response to conA, PHA, PWM, and LPS at any concentration after 12 hours of stimulation (data not shown). Significantly increased IL-18 mRNA expression was detected in response to PWM at 10 µg/ml and LPS at 5 and 10 µg/ml after 24 hours of stimulation (Fig. 3). There was no significant difference in efficiency of PWM and LPS in induction of IL-18 mRNA expression.

Discussion

Numerous studies on porcine cytokine gene expression have been conducted in PBMC and T cells.[1-5] Only some are done in monocytes. These are the studies of IL-1, IL-4, IL-6, IL-10, IL-12, IL-13, IFNγ, tumor necrosis factor alpha, and transforming growth factor beta (TGFβ).[8-13] Stimuli used to induce expression of these cytokine genes are LPS, LTA, peptidoglycan, plasmid DNA, conA, and PHA.[8-13] Among these, LPS is the stimulus that can up-regulate expression of most cytokine genes except IL-4.

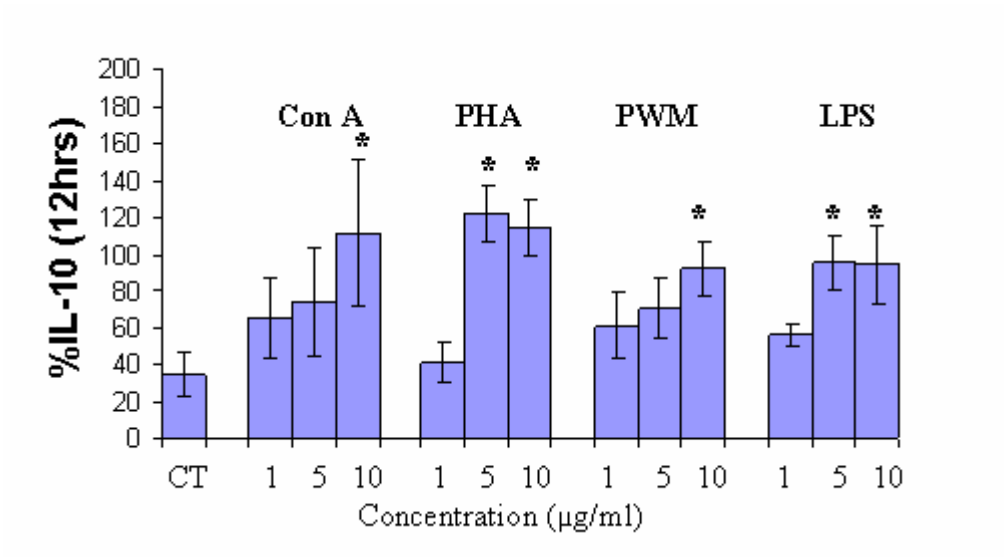

Figure 1. Mean %IL-10 gene expression of monocytes stimulated with conA, PHA, PWM, and LPS for 12 hours. Data were normalized with GAPDH gene expression of the same animal. Error bars indicate the standard error of mean (SEM). * indicate significant difference of mean % IL-10 ($p<0.05$) between stimulated and unstimulated control monocytes (CT)

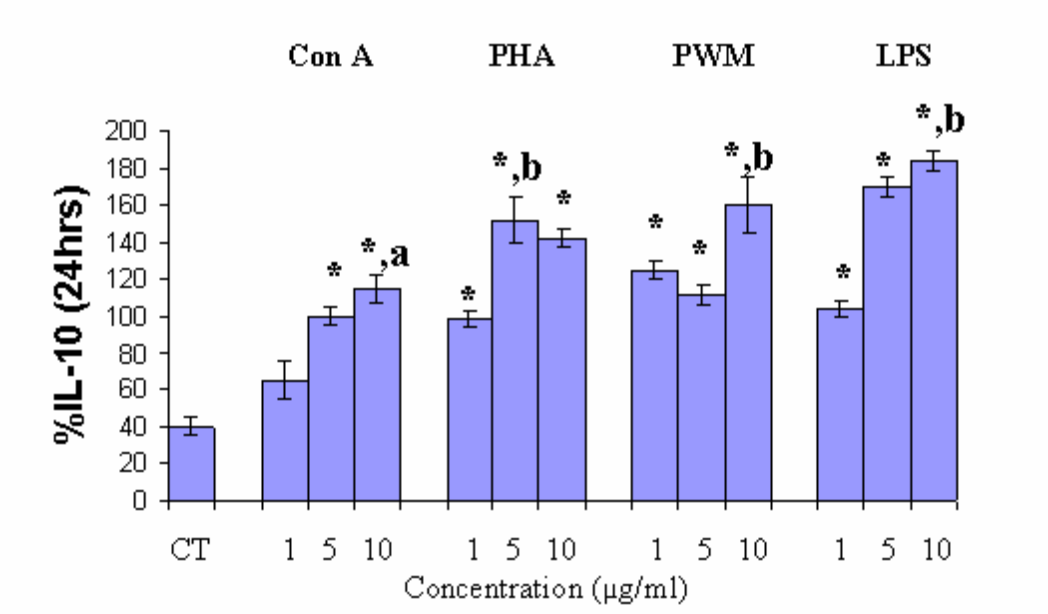

Figure 2. Mean %IL-10 gene expression of monocytes stimulated with conA, PHA, PWM, and LPS for 24 hours. Data were normalized with GAPDH gene expression of the same animal. Error bars indicate the SEM. * indicate significant difference of mean %IL-10 ($p<0.05$) between stimulated and unstimulated control monocytes (CT). Different letters indicate significant difference of mean % IL-10 ($p<0.05$) among stimulated monocytes that showed highest %IL-10 gene expression in each mitogen.

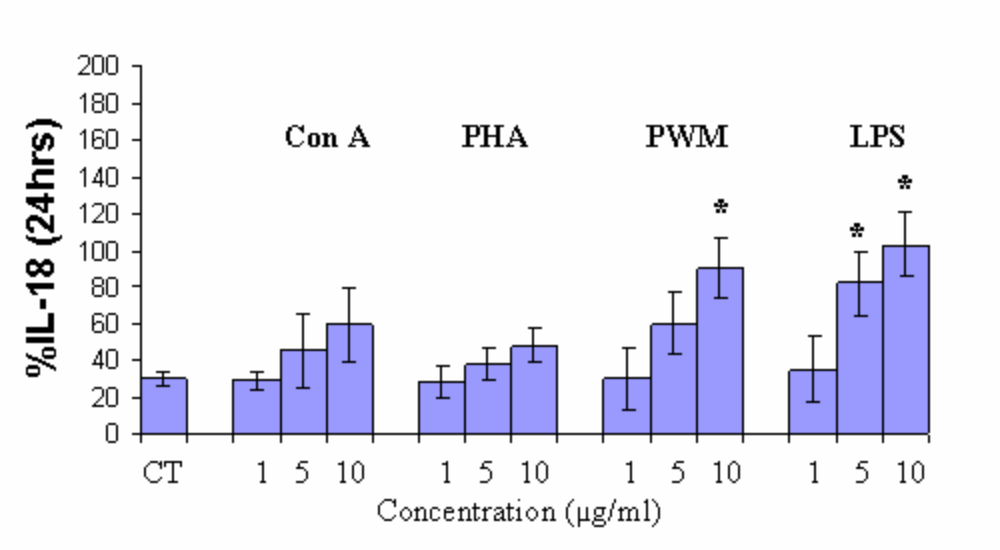

Figure 3. Mean %IL-18 gene expression of monocytes stimulated with conA, PHA, PWM, and LPS for 24 hours. Data were normalized with GAPDH gene expression of the same animal. Error bars indicate the SEM. * indicate significant difference of mean %IL-18 ($p<0.05$) between stimulated and unstimulated control monocytes (CT)

Studies of cytokine gene expression in porcine PBMC and T cells usually use lectin group of mitogens ie. conA, PHA, and PWM as stimulus for positive control of experiment, since this substance up-regulates most cytokine gene expression eg. IL-2, IL-4, IL-10, IL-12 and IL-18[1, 2, 6, 7, 15, 16]. Such lectins have been used only in a few cytokine gene expression studies in porcine monocytes ie. IL-6 and TGFβ.[10, 13] The present work elucidates the efficiency of lectins as well as LPS in inducing IL-10 and IL-18 gene expression in porcine monocytes. It provides new evidence on IL-10 and IL-18 gene expression in porcine monocytes in response to lectin, and also on IL-18 gene expression in response to LPS.

Up-regulation of IL-10 gene expression was detected after 12 hours of monocyte stimulation with conA, PHA, PWM, and LPS at 10 µg/ml, and also with PHA and LPS at 5 µg/ml (Fig. 1). No significant difference in IL-10-inducing efficiency of these mitogens was observed. Higher IL-10 gene expression was achieved when monocytes were stimulated with mitogens for 24 hours. The increased IL-10 gene expression was detected in response to PHA, PWM, and LPS at 1, 5, and 10 µg/ml, and to conA at 5 and 10 µg/ml (Fig. 2). PHA, PWM, and LPS were significantly more potent in inducing IL-10 gene expression than conA when determined at 24 hours of stimulation (Fig. 2). Different levels of IL-10 gene expression was not due to conA-induced monocyte cell death, since the percentage of viable monocytes after conA stimulation was approximately the same as that after PHA, PWM, and LPS stimulation (data not shown).Only PWM (5 µg/ml) and LPS (5 and 10 µg/ml) could up-regulate IL-18 gene expression in porcine monocytes. The up-regulation required 24 hours of stimulation. No significant difference in IL-18-inducing efficiency between PWM and LPS was detected.

This study determined IL-10 and IL-18 gene expression by one-step RT-PCR. This technique allowed comparison of cytokine gene expression in semi-quantitative fashion. The number of PCR cycle used in this study did not reach plateau phase during PCR amplification, thus different levels of cytokine gene expression could be distinguished. One-step RT-PCR was, however, less sensitive in determining gene expression than real-time PCR, and it could not determine

exact RNA quantity. Future studies on cytokine gene expression in porcine monocytes should concern about these limitations of one-step RT-PCR and, if possible, utilize real-time PCR for more precise determination.

This study reveals the potential use of certain lectins and LPS for stimulating IL-10 and IL-18 gene expression in porcine monocytes. The findings may be applied to future IL-10 and IL-18 gene expression studies in which lectins and LPS are used as stimuli for positive control of gene expression.

Acknowledgement

This work is partially supported by the department of biology, faculty of science, Maejo university and the industrial and research projects for undergraduate students (IRPUS) of fiscal year 2007. The author thanks Ms.Surangkanang Yamkanchoo and Ms.Nilobol Chamnan for technical assistance.

References

1 Verfaillie, T, Cox E, To LT, Vanrompay D, Bouchaut H, Buys N, et al. Comparative analysis of porcine cytokine production by mRNA and protein detection. Vet Immunol Immunopathol 2001;1-2:97-112

2 Yancy, H, Ayers SL, Farrell DE, Day A, Myers MJ. Differential cytokine mRNA expression in ne whole blood and peripheral blood mononuclear cell cultures. Immunol Immunopathol 2001;1-2:41-52

3 Feng, WH, Tompkins MB, Xu JS, Zhang HX, McCaw MB. Analysis of constitutive cytokine expression by pigs infected in-utero with porcine reproductive and respiratory syndrome virus. Vet Immunol Immunopathol 2003;1-2:35-45

4 Wattrang, E, Wallgren P, Fossum C. Actinobacillus pleuropneumonia serotype 2 - effects on the interferon-alpha production of porcine leukocytes in vivo and in vitro. Comp Immunol Microbiol Infect Dis 1998;2:135-54

5 Raymond, CR, Wilkie BN. Th-1/Th-2 type cytokine profiles of pig T-cells cultured with antigen-treated monocyte-derived dendritic cells. Vaccine 2004;8:1016-23

6 Charerntantanakul, W, Platt R, Roth JA. Effects of porcine reproductive and respiratory syndrome virus-infected antigen-presenting cells on T cell activation and antiviral cytokine production. Viral Immunol 2006;4:646-61

7 Suradhat, S, Thanawongnuwech R, Poovorawan Y. Upregulation of IL-10 gene expression in porcine peripheral blood mononuclear cells byporcine reproductive and respiratory syndrome virus. J Gen Virol 2003;2:453-9

8 Raymond, CR, Wilkie BN. Toll-like receptor, MHC II, B7 and cytokine expression by porcine monocytes and monocyte-derived dendritic cells in response to microbial pathogen-associated molecular patterns. Vet Immunol Immunopathol 2005;3-4:235-47

9 Charley, B, Laverne S, Lavenant L. Recombinant porcine interferon-gamma activates in vitro porcine adherent mononuclear cells to produce interleukin 1. Vet Immunol Immunopathol 1990;2:117-24

10 Zhou, Y, Scamurra R, Molitor TW, Murtaugh MP. Characterization of transforming growth factor-beta 1 gene expression in porcine immune cells. Mol Immunol 1992;7-8:965-70

11 Hofstetter, C, Kleen M, Habler O, Allmeling AM, Krombach F, Zwissler B. Recombinant human interleukin-10 attenuates TNFalpha production by porcine monocytes. Eur J Med Res 1998;6:299-303

12 Basta, S, Knoetig SM, Spagnuolo-Weaver M, Allan G, McCullough KC. Modulation of monocytic cell activity and virus susceptibility during differentiation into macrophages. J Immunol 1999;7:3961-9

13 Schwager, J, Schulze J. Maturation of the mitogen responsiveness, and IL2 and IL6 production by neonatal swine leukocytes. Vet Immunol Immunopathol 1997;1-2:105-19

14 Johansson, E, Domeika K, Berg M, Alm GV, Fossum C. Characterisation of porcine monocyte-derived dendritic cells according to their cytokine profile. Vet Immunol Immunopathol 2003;3-4:183-97

15 Dufour, V, Arnauld C, Lantz O, Peguillet I, Morvilliers K, Emmanuel A, et al. Quantification of porcine cytokine gene expression using RT-PCR, homologous internal control and chemiluminescence for microplate detection. J Immunol Methods 1999;1-2:49-60

16 Andersson, M, Berg M, Fossum C, Jensen-Waern M. Development of a microarray for studying porcine cytokine production in blood mononuclear cells and intestinal biopsies. J Vet Med A Physiol Pathol Clin Med2007;3:161-8