

## Development of a novel antigen capture-ELISA using IgY against porcine interleukin-6 and its application

Deog Yong Lee, Young Wook Cho, Sang Gyun Kang, Sung Jae Shin, Han Sang Yoo\*

*Department of Infectious Diseases, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Seoul, 151-742, Korea*

Interleukin-6 (IL-6) is introduced as a marker of disease. At present, a variety of method may be used to quantify expression of this protein. Antigen capture-ELISA is a sensitive and accurate quantification method previously used with ovine, rat, and human IL-6 proteins. However, it has never been reported to quantify porcine IL-6 protein using capture ELISA. In this study, we generated and characterized a set of IgY and mono-specific polyclonal antibodies to recombinant porcine IL-6 (rpIL-6), and combining these with a sensitive and specific capture-ELISA for a diagnostic purpose. cDNA encoding the mature protein coding region of porcine IL-6 was cloned and expressed with pQE-30UA expression vector. rpIL-6 was then expressed and purified by using Ni-NTA resin. Protein mass of 24 kDa was found with SDS-PAGE and the identity of the protein was confirmed by Western-blot. Production of polyclonal antibodies against rpIL-6 was performed using the purified rpIL-6 in mice and hens. An antigen capture-ELISA was developed with the antibodies after their extraction. To compare the IL-6 level in the different sanitary state of farms, pig sera were randomly collected and concentration of IL-6 in the sera was measured with the antigen capture-ELISA. The capture-ELISA with the optimal concentration of antibodies, in this study, was able to detect about 10 ng/ml of rpIL-6. IL-6 levels determined with the capture-ELISA in pig sera showed positive correlation with the sanitary states of the farms. These results suggested that the developed antigen capture-ELISA could be a good tool for the screening of microbial infection in pig farms.

**Key words:** Antigen capture-ELISA, porcine interleukin-6, IgY, protein expression

## Introduction

Interleukin-6 (IL-6) is a 21 to 28 kDa glycoprotein [23, 30] and a multifocal cytokine, produced by both lymphoid and non-lymphoid cells [18]. IL-6 plays an important role in immune response, hematopoiesis, and acute-phase reaction. IL-6 induces B cell proliferation and differentiation [12,13, 27], antibody production [11], and T-cell activation and differentiation [35]. In addition, IL-6 stimulates hematopoietic stem cells and macrophage differentiation in several human and murine cell lines. Also, a variety of acute-phase proteins, such as fibrinogen,  $\alpha_1$ -antichymotrypsin,  $\alpha_1$ -acid glycoprotein, and haptoglobin, are induced by IL-6 [10].

This study introduces the use of IL-6 as a marker of disease in swine. The appearance of IL-6 positive pigs coincided with the onset of clinical signs of disease and increased body temperature associated with acute bacterial infection [3]. In challenge studies of SIV-vaccinated pigs, levels of IL-6 with IFN- $\alpha$  and TNF- $\alpha$  were correlated with both clinical and viral protection [17]. Used as a marker of disease, measurement of IL-6 concentration in serum predicts the disease status of pigs or farms.

Antigen capture-ELISA is a sensitive and accurate quantification method [24] which usually uses monoclonal antibodies to increase sensitivity. However, monoclonal antibody preparation for capture-ELISA requires great skill and laborious job. Therefore, for cheap and easy preparation, we used IgY as a capture-antibody instead.

IgY is the typical low-molecular-weight (LMW) egg yolk serum antibody of birds, reptiles, amphibians and lungfish, whereas IgG occurs in mammals [8]. Because of evolutionary difference, chicken IgY reacts with more epitopes on a mammalian antigen, producing an amplification of the signal. IgY also has the advantage in that it avoids the interference in immunological assays caused by the complement system, rheumatoid factors, anti-mouse IgG antibodies or human and bacterial Fc receptors [2].

Quantification of IL-6 protein using capture ELISA has been done using ovine, rat, and human IL-6 [9,24,28]. Detection of porcine cytokines using capture-ELISA has

\*Corresponding author  
Tel:+82-2-880-1263; Fax: +82-2-874-2738  
E-mail: yoohs@snu.ac.kr

only been performed with IFN- $\gamma$ , IL-8 and IL-18 [21, 26,33]. To our knowledge this is the first study to use the ELISA capture method to quantify concentrations of porcine IL-6, which were then used as a marker for disease.

This study generated and characterized a set of IgY and polyclonal antibodies to recombinant porcine IL-6 (rpIL-6), and then combined these antibodies to develop a sensitive and specific capture-ELISA for the diagnosis of a farm's sanitary state.

## Materials and Methods

### Production of recombinant pig IL-6

**Cloning of cDNA encoding mature protein:** Total RNA was extracted from PBMCs using Trizol reagent (Gibco, USA) and chloroform after stimulation with 20  $\mu$ g/ml of phytohemagglutinin (PHA, Invitrogen, Carlsbad, USA) for 4 hr. Single-stranded cDNA was synthesized using the Superscript preamplification system for First strand cDNA synthesis kit (Gibco, USA). PCR primers were designed to amplify the mature protein-coding region of IL-6, without a signal peptide sequence (F, 5'-GAACGC CTGGAAGAAG ATGCC-3'; R, 5'-CTACATTATCCGAATGGCCCTC-3'). Purified PCR products were cloned into the pQE30-UA expression vector (Qiagen, Germany). Sequence identity of the cloned cDNA encoding the pIL-6 gene was confirmed using an automated DNA sequence (ABI PRISM 377xL, Perkin Elmer, USA).

**Screening of clones producing porcine IL-6:** A single colony was inoculated into 1.5 ml of LB broth containing 100  $\mu$ g/ml of ampicillin and 25  $\mu$ g/ml of kanamycin and was then grown at 37°C. Five hundred  $\mu$ l of this culture was used to inoculate a 10 ml of pre-warmed medium with the antibiotics listed above and cultured at 37°C for 100 min at 300 rpm until the OD<sub>600</sub> reached 0.6–0.7. After 5 hours culture in the presence of 1 mM of IPTG, the cells were harvested by centrifugation at 15,000  $\times$  g for 10 minutes.

Identification of porcine IL-6 producing clones was performed by SDS-PAGE analysis of uninduced and induced cell lysates followed by Western-blot using an antibody against rpIL-6 (Endogen, USA).

Western-blot was performed with purified anti-pig IL-6 antibody (Endogen, USA). Briefly, 5  $\mu$ l of lysates were loaded into a 12% SDS-PAGE and run under reducing condition. The separated lysates were then electro-blotted onto a nitrocellulose membrane and blocked with 3% gelatin in phosphate buffered saline (PBS; pH7.4) also containing 0.05% Tween 20. After treatment with anti-pig IL-6 antibody, the blot was incubated for 1 hr at room temperature with anti-mouse-IgG-HRP (BioRad) then washed and visualized using HRP substrate reagent (BioRad, USA).

**Protein purification:** The cells producing rpIL-6 were cultured in 500 ml of media and harvested by centrifugation. The cells were resuspended and then lysed with lysis buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-Cl, 6 M GuHCl, pH 8.0). The cell suspension was additionally lysed by sonication and then incubated with 4-volumes of Ni-NTA resin for 1 hr. This lysate-resin mixture was loaded into a column and washed with washing buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-Cl, 8 M Urea, pH 6.3). Protein elution was done using elution buffer with serial pH from 8.0 to 4.5 (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-Cl, 8 M Urea). Fractions from each elution were analyzed by SDS-PAGE and Western-blot assay to show purity and specificity, respectively. Concentration of the purified protein was measured using protein assay kit (Bio-Rad, USA) with bovine serum albumin (Bio-Rad, USA) being used as a standard protein.

### Production of polyclonal antibodies against rpIL-6

**Immunization of mice and hens with rpIL-6:** Four week-old female mice (ICR) were immunized by injection of 500  $\mu$ g/ml of rpIL-6. At first, 200  $\mu$ l of rpIL-6 were injected subcutaneous with the same volume of Freund's complete adjuvant (Sigma, USA). The second and the third boosting were done 10 days after each immunization. Freund's incomplete adjuvant was used for second and third immunization.

Twenty-four week old white egg laying hens were used to produce IgY antibody. Hens were injected intramuscularly with 500  $\mu$ g/ml of rpIL-6 emulsified with Freund's complete adjuvant. The second and the third were carried out at 10 day after each immunization with Freund's incomplete adjuvant. Eggs were collected 7 days after the third immunization, to extract IgY antibodies.

**Antibody extraction:** Mouse whole blood was obtained from abdominal vein and incubated at 4°C overnight. Mouse sera was then collected by centrifugation and stored at –20°C until use.

Egg yolk antibody was extracted from eggs collected weekly after immunization [32]. Egg yolk was separated from the egg white and homogenized with an equal volume of PBS (pH 7.2). Homogenized egg yolk was mixed with an equal volume of chloroform and incubated at room temperature for 2 hr. The supernatant was separated by centrifugation at 5,500 rpm for 10 min and collected. Finally, extracted IgY was filtered using a membrane filter with 0.45  $\mu$ m pore size and stored at –20°C until use. Specificity of the antibodies were confirmed by ELISA with different porcine cytokines such as IFN- $\gamma$ , GM-CSF.

### Titration of antibodies to rpIL-6

Optimization of the antibody titer was conducted using a check board titration of ELISA. In each microplate well,

**Table 1.** Sanitation check lists of pig farms

Title		Check Points	Score <sup>a</sup>
Biosecurity	Isolation	Is it distant from the nearest swine herd?	
		Is it distant from the road to the nearest swine building above 100m?	
	Entrance	Is there a separate change area for staff or visitors?	
		Is quarantine area always used for incoming stock?	
	Building	Are cats or dogs allowed into building?	
		Are rodents, other wild life, or birds present in buildings?	
Management	Feeding	Is there a chance of cross-contamination at feed delivery	
	Ventilation	Is there a ventilation system for air condition?	
	Shipping	Is own truck/trailer used for shipping?	
	Dead stock	Is disposal by burial, composting or dead stock service?	
	Manure Removal	Is there a risk of yard contamination by out side hauler?	
Health issue	Outbreak	Is there an experience of outbreak?	
	Vaccine	Is there a good vaccine program?	
	Clinics	Is there a clinical symptom related disease?	
Sum			

<sup>a</sup>Grades from 5 to 0. Total score was 70.

one-hundred µl of purified rpIL-6, ranging from 580 µg to 1.09 ng, was coated by overnight incubation at 4°C. After unbound antigens were removed by washing, the wells were each blocked with 100 µl of 1% bovine serum albumin (BSA) in PBST per well. One-hundred µl of mouse serum and egg yolk were 4-fold serial diluted with PBST and incubated for 1 hr at 37°C. Plates were then washed three times with PBST. Horse radish peroxidase- conjugated goat-anti mouse IgG (Bio-Rad, USA) or horse radish peroxidase-conjugate rabbit IgG fraction to chicken (Cappel, USA) were added to the microplates with 1 : 2,000 dilution and incubated for 1 hr at 37°C. The plates were then washed as above. 2,2'-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) substrate was added and the optical density value was determined at 405 nm using a microplate reader after 30 min.

#### Antigen capture-ELISA

The egg yolk was 1 : 1,000 diluted in coating buffer and coated by incubation at 4°C overnight. Plates were blocked and washed as described above. Purified rpIL-6 was 10-fold serial diluted in PBST and 100 µl of diluted sample was added to each well and incubated for 1 hr at 37°C. Mouse anti-rpIL-6 serum was used to detect captured rpIL-6 (diluted 500:1 in PBST containing 1% gelatin). Horseradish peroxidase-conjugated goat-anti mouse IgG (BioRad, USA) was used as the secondary antibody and developed with ABTS. Microplates were read at 405 nm by the optical density reader. Detection limit of the ELISA was determined at the lowest concentration of rpIL-6 shown P/N ≥ 2.

#### Pig's sera

Serum samples were collected randomly from the middle

region of Korea from 5 farms showing different sanitary states between July to October 2003. The sera were stored at -20°C prior to use. The ages of these pigs varied and there was no association between collection of the samples and the presence of a known recent disease. However, grades of the sanitary states were evaluated based on our guide- lines (Table 1). Concentrations of IL-6 in the sera were measured with the developed antigen capture-ELISA after being 100-fold diluted with PBST.

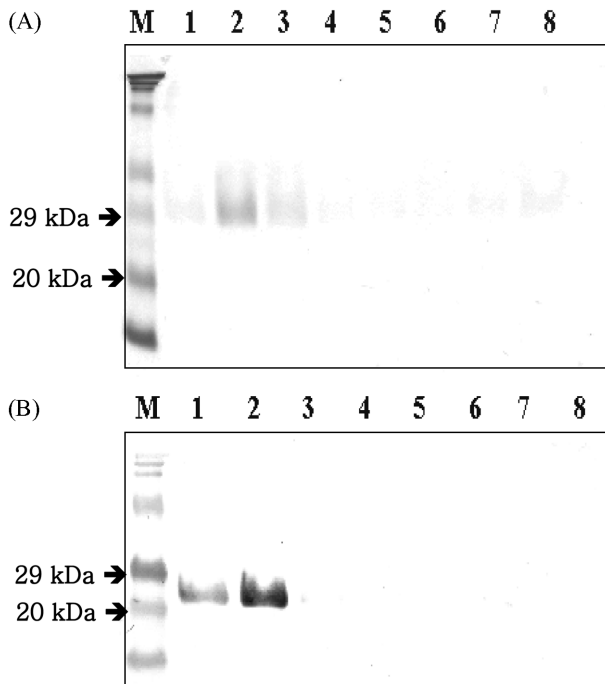
## Results

#### Expression and purification of rpIL-6

Transformants harboring inserted cDNA, (the 552 base pair encoding the mature protein region of IL-6), were selected by colony PCR and restriction enzyme digestion (data not shown). A twenty-four kDa component, the expressed recombinant porcine IL-6 (rpIL-6) was identified by 12% SDS-PAGE and Western-blot (Data not shown). rpIL-6 expressed in *E. coli* M15 by IPTG induction was purified using Ni-NTA resin. The molecular mass of this protein was 24 kDa in SDS-PAGE and the identity of the protein was confirmed by Western-blot (Fig. 1).

#### Titration of antibodies to rpIL-6

Extracted antibodies had a specificity to rpIL-6 but not rpIFN-γ and rpGM-CSF which were expressed and purified by the same method of rpIL-6. Indirect ELISA was used to titrate mouse IgG and IgY antibodies to rpIL-6. Sixteen-fold diluted IgY responded up to 1 ng/ml of rpIL-6 and 1 : 64~1 : 4,096 dilution of IgY was up to 30~250 ng/ml. Based on the results, optimal IgY concentration was about 1 : 1,000 dilution (Fig. 2). Four-hundred fold diluted mouse



**Fig. 1.** Analysis of purified IL-6 after expression in *E. coli* by SDS-PAGE (A) and Western-blot (B). M, standard molecular weight marker; Lanes 1-8: eluted fractions from 1-8. Protein size was approximately 24 kDa as shown by SDS-PAGE. IL-6 Identity of IL-6 was confirmed by Western-blot using a polyclonal antibody against IL-6.

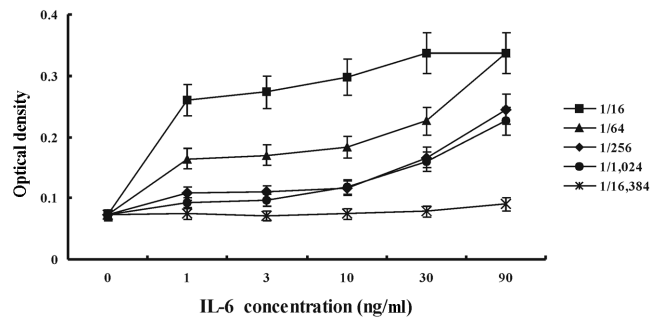
serum responded up to 1 ng/ml of rpIL-6 and 1 : 1,600~100,000 dilution of IgG was effective to 30~760 ng/ml. Optimal IgG concentration was about 1 : 400 dilution (Fig. 3).

#### Antigen capture-ELISA

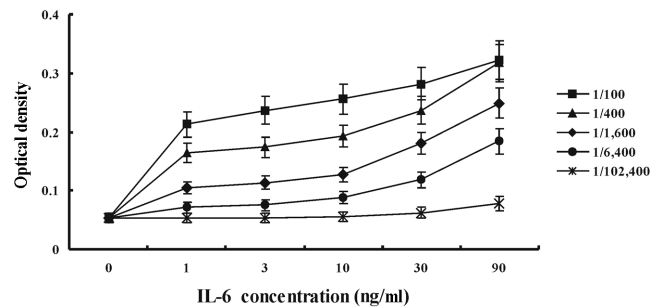
Condition of capture-ELISA was optimized with mouse IgG and IgY antibodies on the basis of the titration. To organize antibody titers, the optimal condition of antigen capture-ELISA was followed: chicken IgY, 400~1,000 dilution, 25~50 µg/ml; mouse IgG, 1 : 400 dilution; horse radish peroxidase-conjugated goat-anti mouse IgG, 1 : 2,000. With those optimized conditions, the optimal antigen capture-ELISA could reliably detect at about 10 ng/ml of rpIL-6 (Fig. 4).

#### Measurement of pIL-6 in swine sera with the ELISA

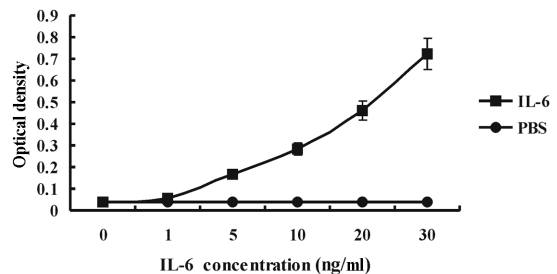
Antigen capture-ELISA was applied to detect pIL-6 in porcine sera. In one-hundred samples of total serum, most samples were below the 10 ng/ml of pIL-6 (67%). However, a number of samples were in the 1,000 to 10,000 ng/ml range (16%). 27 µg/ml was the maximum concentration of pIL-6 detected in the sera. A- and E- farms were lower sanitary states on our guidelines, which were 20-30 scores. C- and D- farms were higher states, which were 50-60 scores and B-farm was middle states, relatively. However, the sanitary states of B- farm closed to C- and D- farms,



**Fig. 2.** Antibody titer of chicken IgY against rpIL-6. Concentration of IL-6 protein ranged from 0 to 250 ng/ml and IgY was 4-times serial diluted. IgY was up to 1 : 1,024 dilutions for reaction with IL-6 protein.



**Fig. 3.** Antibody titer of mouse IgG against rpIL-6. IL-6 and IgG were diluted and IgG was up to 1 : 10,240 dilution ratios for reaction with IL-6 protein.

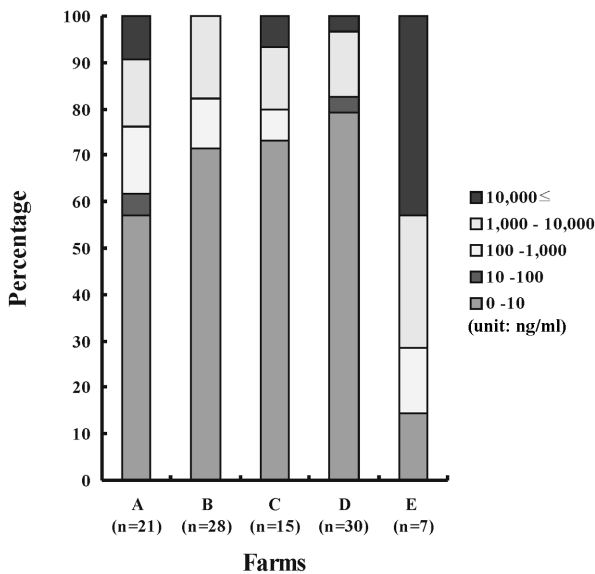


**Fig. 4.** Antigen capture-ELISA for detection of rpIL-6. Concentration of rpIL-6 ranged from 0 to 30 ng/ml/ and was normalized by PBS. Developed capture-ELISA detected about 10 ng/ml of rpIL-6 concentration.

which was about 40 scores. IL-6 levels in pigs from farms A and E were higher than those from other farms (Fig. 5). This distribution positively correlated to the sanitary states of the farms that provided the porcine sera.

#### Discussion

This paper describes the purification of rpIL-6, the production of antibodies and the development of a sensitive antigen capture-ELISA for porcine IL-6 for clinical diagnostic purpose.



**Fig. 5.** Distribution of IL-6 in pig sera. Sera were collected from the different farms. (A-, B-, C-, D- and E-farms) Each farm has different management system and methods of disease control. It was different distribution to detect IL-6 concentration in the each farm.

To express the porcine IL-6 in *E. coli*, cDNA encoding mature protein was amplified and transformed into *E. coli*. of the transformants, positive clones were screened by growth on LB plates containing appropriate antibiotics and colony PCR. To confirm the identity of the cloned gene, restriction enzyme analysis and sequencing of the plasmid DNA were performed because of appearance of false positive in colony PCR. Purified rpIL-6 was identical in size with a previous report [35]. The identity of the protein was also confirmed by Western-blot with a polyclonal antibody after expression in *E. coli* by IPTG induction.

The expressed protein, rpIL-6, in *E. coli* was purified using Ni-NTA resin and elution buffer with pH gradients. The concentration of purified rpIL-6 was 580 µg/ml. To improve the purification efficacy, washing with appropriate pH and imidazole concentration were the most important factors [8,29]

Five to ten ml of egg yolk was harvested from each egg with the approximate concentration of IgY reaching 20 mg/ml. One to two hundred µl of serum was obtained per mouse, with approximately 10% constituting the specific antibody. This data shows that 100~200 mg/ml of IgY per yolk was produced and this value is similar to another report [25]. Furthermore, pIL-6 specific IgY production might be estimated at 2 to 20 mg per yolk, because the constitution of specific IgY is between 2-to10 % [34].

Antibody titer against rpIL-6 was evaluated by direct-ELISA. Mouse serum responded effectively on coated rpIL-6 at 1 : 400 ratio and chicken IgY at 1 : 1,000 ratios. In the optimization of this capture-ELISA, there were differences

in mouse IgG titer but no difference of tendency for using first antibody in capture-ELISA. However, there was a difference of tendency in IgY titer for using coated protein in a microtiter plate. Optimal dilution for IgY coating was 1 : 400~1 : 1,000 ratio. Low dilution ratio of IgY was less detective than optimal concentration as well as high ratio. In capture-ELISA, monoclonal antibody was used generally with 300 ng/ml of concentration [22]. Because the specific antibody portion is less than 5% in produced polyclonal antibody [5], optimal concentration of IgY coated was 25~50 µg/ml. This result revealed that the concentration of specific antibody is 12.5 µg/ml on coated IgY.

High concentration of urea may interfere with the ELISA cross-reaction [1]. In this study, the concentration of urea was diluted well below the minimum level (0.1 M) by dilution of the purified rpIL-6. Therefore, the effect of urea should not be seen.

Most versions of capture-ELISA have used a monoclonal antibody for the capture-antibody and a biotinylated antibody to increase detection limit [6,9,26,33]. However, pico-gram levels in any case of using monoclonal antibody without biotinylation for detection antibody have not been reached [21]. That reports suggest that modification of the detection antibody plays an important role in the sensitivity of capture-ELISA. However, there were some exceptions [22,24]. The sensitivity could be increased by other detection antibody modification, such as immunopurification [20] and by using an IL-6 dependent cell line [16]. Although IgY was used instead of a monoclonal antibody for the capturing antibody, the sensitivity could still reach the nano-gram level. Monoclonal antibody usefulness stems from three characteristics: specificity of binding, homogeneity, and capacity for unlimited production. In practice, however, producing the right monoclonal antibody is often a difficult and laborious job [4]. IgY has the amount of specific extractable antibody in egg yolk than in rabbit in the same period, besides the advantage of a non-invasive antibody sampling [31]. An egg contains 100~150 mg of IgY per yolk [25]. Furthermore, the detection of capture-ELISA using IgY coating reached the same level as using a monoclonal antibody without biotinylation has the detection antibody [21]. Our results correctively matched with previous reports as described above.

Detection level of pIL-6 was increased via dilution ratio rather than non-diluted porcine sera and reached the critical point at 100-fold dilution. Interference with some serum components to perform the antigen capture-ELISA is possible [36]. However, high level of pIL-6 was detected in many samples (33%) by developed capture-ELISA and it revealed that it is detectable IL-6 secretion in pig.

Pig sera were collected from 5-farms showing different sanitary states. These farms were different in their management and control of disease. Distribution of pIL-6 concentration was positively correlated with the sanitary

status of farms. A- and E-farms have the similar conditions of old-fashioned equipment and have several problems with chronic infectious diseases such as respiratory diseases and old-traditional management. They are small-scale farms with 1,200 pigs in A-farm and 1,500-2,000 pigs in E-farm. However, the farmers have difficulty because of their old age and deficient education. pIL-6 were relatively high distributed from 2,500 to 11,000 ng/ml in mean values. Although they have similar conditions and symptoms, E-farm detected a higher concentration. This result indicates that A- and E-farms have on-going infection and that in addition, E-farm might have a new infection recently.

C- and D-farms are similar to each other in their conditions and management, with newly introduced 3,000-3,500 pigs and good equipments. Also, these farms have a larger scale, more modernized than the A- and E-farms. Here samples distributed lower than the 10 ng/ml of pIL-6 indicating that the control of disease was well performed. Finally, B-farm has a good management system and has about 3,000 head of pigs. However, there are sometimes outbreaks of pluropneumoniae and diarrhea. Some samples distributed relatively high between 100 to 10,000 ng/ml of pIL-6 which may indicate an infection is just beginning. These results demonstrate the clinical diagnostic use of antigen capture-ELISA for pIL-6.

It is necessary to study protein characteristics for improving immunogenicity by using native protein rather than denatured [15] and IL-6, like other cytokines are critically regulated secretion and inactivation by lymphocytes [19].

## Acknowledgments

This research was supported by BioGreen 21, BrainKorea 21 and the Research Institute for Veterinary Science (RIVS), Seoul National University, Korea.

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