Detection of Serum IgE Specific to Mite Allergens by Immuno-PCR

*Kyung-woo Lee[1], Byung-ung Hur[1], Kaw-yan Chua[2,3], I-chun Kuo[4], Suk-yoon Song[1] and Sang-hoon Cha[1,5,6]**

[1]Division of Molecular and Medical Biotechnology, College of Bioscience and Biotechnology, Kangwon National University, Chuncheon, Korea, [2]Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore, [3]Member of Immunology Programme, National University of Singapore, [4]Singapore Institute for Clinical Sciences, Agency for Science, Technology and Research, Singapore, [5]Institute of Bioscience and Biotechnology, Kangwon National University, [6]IG Therapy Co., Rm 112, Nong 3rd Building, Kangwon National University, Chuncheon, Korea

Background: Although a skin test is the primary option for detecting allergen-specific IgE in clinics, the serum IgE immunoassay is also important because it allows for the diagnosis of allergy without any accompanying adverse effect on the patient. However, the low detection limit of IgE levels by immunoassay may restrict the use of the method in some occasions, and improving its sensitivity would thus have a significant implication in allergy-immunology clinics. **Methods:** In this study, we attempted to detect specific serum IgE by using immuno-polymerase chain reaction (IPCR) which combines the antigen-antibody specificity of enzyme-linked immunosorbent assays (ELISAs) with the amplification power of PCR. **Results:** Our results demonstrated that Blo t5-specific serum IgE can be detected by IPCR with a 100-fold higher sensitivity than ELISA, and cross-reactivity of serum IgE to other mite allergens is able to be analyzed by using only 0.3 μl of serum sample. Use of real-time IPCR seemed to permit more convenient determination of specific serum IgE as well. **Conclusion:** We believe that IPCR can serve as a valuable tool in determining specific serum IgE, especially when the amount of serum sample is limited.

[Immune Network 2008;8(3):82-89]

INTRODUCTION

Atopic allergic diseases affect 15% to 25% of the general population, and the prevalence of atopy and asthma are increasing in all developed countries for unknown reasons (1), implying that the importance of more accurate diagnosis is more demanding in allergy clinics. Skin testing is the primary and most sensitive method to detect allergen-specific IgE, and is considered as a clinical standard because of its routine usage in clinics for a long time. However, training and experience are required for both accuracy and consistency of the testing (2,3). In addition, skin testing cannot be performed in some occasions especially when a patient is taking a medication such as an anti-histamine or has extensive skin disease with a possible risk for systemic reaction such as anaphylaxis. Very young children, the elderly and women during pregnancy are not appropriate subjects for testing, either (4).

Allergen-specific IgE also can be detected by immunoassays which permit the detection of specific serum IgE when the skin testing is impractical to perform. Immunoassays have been used for many diagnostic applications since the 1960s and a number of ways to detect a binding signal between analytes and antibodies have been developed. Especially, the use of labeled antibodies makes immunoassays more simple and convenient (5). For instance, the radioallergosorbent test

Received on August 12, 2008. August 22, 2008. Accepted on August 29, 2008.
*Corresponding Author. Tel: 82-33-250-6485; Fax: 82-33-253-6485; E-mail: chash@kangwon.ac.kr
This work was partially supported by the sabbatical research grant of Kangwon National University and by the Program for the Training of Graduate Students in Regional Innovation which was conducted by the Ministry of Commerce, Industry and Energy in South Korea.

Keywords: Blo t 5, Der p 1, Der p 2, IgE, allergy, immuno-PCR, real-time PCR
Abbreviation: phosphate buffered-saline (PBS), immuno-polymerase chain reaction (IPCR), glutathione S-transferase (GST), phosphate-buffered saline containing 0.05% Tween-20 (PBST), Enzyme-linked immunosorbent assay (ELISA), polyclonal antibody (pAb)

(RAST) is taking advantage of radioisotopic labels such as ^{125}I to measure specific serum IgE (6). Recently, however, enzymes such as horseradish peroxidase and alkaline phosphatase have also been widely used as labels in the CAP system (7) because of easy handling as well as increased detection limits through the use of fluorogenic (8) or enzyme amplification methods (9,10). The CAP system is the most widely used currently (11). However, they are generally less sensitive than the skin prick test, and it had been estimated through comparison studies that the sensitivity of immunoassays is about 70～90% of epicutaneous skin testing (12). Therefore, a discrepancy in diagnosis can occur in such a way that the skin test is positive, but the immunoassay is negative, mainly due to limitations of the immunoassay rather than problems with the skin test (13). Since the low detection limit of IgE by immunoassay appears to be problematic, improving the sensitivity of the immunoassay would have a significant implication in allergy-immunology clinics, particularly in the evaluation of asthma, rhinitis, atopic dermatitis or systemic anaphylaxis sensitized to drugs, latex or bee venom.

Previously, Sano et al. had developed the immuno-PCR (IPCR) method, extending the scope of PCR for the highly sensitive detection of proteins with as few as 10～580 molecules of analyte (14). Taking advantage of combining the molecular specificity of antibodies in enzyme-linked immunosorbent assays (ELISAs) with the amplification power of the PCR, the detection limit of a given ELISA can be enhanced 100～10,000 fold by IPCR. The key feature of IPCR is the use of a specific antibody coupled to a reporter DNA molecule either by direct conjugation or *via* streptavidin-biotin interaction (15,16), which enables reflecting the amount of target protein bound by the antibodies to the amount of PCR product formed. The resulting PCR products are assessed either by gel electrophoresis (14,17), PCR-ELISA (18), or much more conveniently by using real-time PCR in which the amount of amplified DNA could be quantified sensitively and accurately over a wide concentration range (16,19). Currently, this method has become a well-established technique frequently applied in both fundamental and applied immunological research, and various target molecules including cytokines (20-22), viral or bacterial proteins (23-26) or specific serum IgG (27), yet the detection of specific serum IgE antibodies to allergens has not been attempted so far. In this study, we describe the application of the IPCR assay in detecting specific-serum IgE and demonstrate that serum IgE specific for *Blomia tropicalis* group 5 allergen (Blo t 5), *Dermatophagoides pteronyssinus* group 1 (Der p 1) or 2 allergens (Der p 2) can be detected with high sensitivity.

MATERIALS AND METHODS

Preparation of sera and recombinant allergens

Six sera from patients allergic to Blo t 5 and three cord blood sera, and recombinant mite allergens (Blo t 5, Der p 1 and Der p 2) (28) were kindly provided from Dr. Chua at National University of Singapore.

Enzyme-linked immunosobent assay (ELISA)

Maxisorp 96-well plates (Nunc, Denmark) coated with 50 μl of 10 μg/ml recombinant Blo t5 or glutathione S-transferase (GST). After coating at 4°C overnight, each well was washed three times with phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBST), and blocked with 200 μl blocking buffer (PBS with 3% dry milk, pH 7.0) at room temperature for 1 h. After washing three times with PBST, 50 μl of sera diluted appropriately with blocking buffer was added to each well, and incubated for 1 h at room temperature. After washing three times with PBST, 50 μl of 40 ng/ml biotinylated goat anti-human IgE antibody (KPL, USA) in blocking buffer were added to each well and the plate was further incubated for 1 h at room temperature. Washing was performed three times with PBST again to remove unbound IgE, and streptavidin-horse radish peroxidase (HRPO) (Amersham Pharmacia, Sweden) conjugated antibody 1:2000 dilution in blocking buffer was added to each well and the plate was further incubated for 1 h at room temperature. The signal was visualized by adding 50 μl of the 3.3',5.5'-tetramethyl benzidine (TMB) substrate (Sigma Co.). The enzymatic reaction was terminated by adding an equal volume of 1 N HCl into each well. Absorbance at 450 nm was measured by using an ELISA reader (Biorad).

Preparation of reporter DNA

Biotinylated double-stranded DNA (409 bp in size) was generated by PCR amplification of rice soluble starch synthease cDNA with a sense primer (5'-CTAGTGAGCAGGAGTCTGAGATCATG-3', corresponding to nt 451～476) and a biotinylated anti-sense primer (5'-GAGTGTGTATCTGAACTGATTATCGCC-3', corresponding to nt 836～860). PCR was performed under the following condition: 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each deoxyribonucleotide, 0.1 μM of each primer, 1.5 U of Ex-*Taq* polymerase (Takara) and 10 ng of pET22b containing full length

rice soluble starch synthease cDNA (IG Therapy Co., South Korea). The temperature profile was as follows: 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min. After PCR amplification, the PCR product was purified using PCR cleanup kit (Promega, USA).

Immuno-PCR

The immuno-PCR (IPCR) was performed as described in the previous report with slight modifications (29) (Fig. 1). Heat stable TopYield™ Strips (Nunc, Denmark) were coated with 30 μl of 5 μg/ml recombinant Blo t 5, Der p 1, Der p 2 or GST per well. After coating at 4°C overnight, each well was washed three times with phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBST), and blocked with 150 μl blocking buffer (PBS with 3% dry milk, 5 mM EDTA and 1 μg/ml calf thymus DNA (Sigma Co., USA)) at room temperature for 1 h. After washing three times with PBST, 30 μl of sera diluted appropriately with PBS containing 3% dry milk (PBSM) was added to each well, and incubated for 1 h at room temperature. Normal control serum was purchased from Sigma and included in the experiments. After washing three times with PBST, 30 μl of 40 ng/ml biotinylated goat anti-human IgE antibody (KPL, USA) in PBS containing 0.5% bovine serum albumin (Sigma Co.) and 5 mM EDTA (PBSBE) were added to each well and the plate was further incubated for 1 h at room temperature. Washing was performed three times with PBST again to remove unbound IgE, and 100 ng/ml streptavidin (Sigma) (30 μl/well) was added into each microwell. After incubation at room temperature for 30 min, the strips were washed three times with PBST, 6.5 ng/ml of the biotinylated DNA (30 μl/well) was added, and the mixture was incubated at room temperature for 30 min. The stripes were washed six times with PBST and four times with sterile dH_2O to remove unbound biotinylated DNA. The PCR was performed using a Perkin-Elmer Cetus 9600 instrument by adding 30 μl of PCR mixture to each well. PCR reaction mixture contains 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM MgCl2, 0.2 mM deoxyribonucleotide (0.2 mM each), 0.2 mM of sense primer (5'-CTAGTGAGCAGGAGTCTGAGATCATG-3') and anti-sense primer (5'-GAACTGATTATCGCCAAAAGCACC-3'), and 1.5 U of GoTaq DNA polymerase (Promega). The temperature profile was as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min; and final extension at 72°C for 10 min. Ten microliters of PCR product (371 bp) was analyzed using a 1.5% (w/v) agarose gel, followed by staining with ethidium bromide. DNA bands in the gel were visualized on a UV transilluminator and digitally photographed. Brightness/contrast of the resulting gel images was arbitrarily adjusted until background signal became invisible.

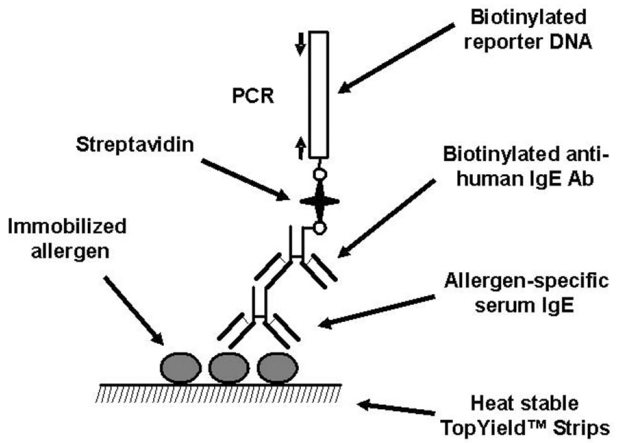

Figure 1. Schematic representation of the IPCR assay used in this study. Heat stable TopYield™ Strips were used to immobilize allergens, and a pre-determined concentration of streptavidin was used as the bridge to link biotinylated anti-human IgE pAb with biotinylated reporter DNA from rice starch synthase cDNA. PCR amplification of the DNA was performed either by using a conventional PCR or real-time PCR machine.

Real-time IPCR

Real-time IPCR was performed with ABI PRISM 7000 (Applied biosystems, USA) as above except that SYBR premix Ex-*Taq* (Takara, Japan), 0.1 μM sense primer (5'-GCTCTTGCTCTT-CGTGGTCATC-3') and 0.1 μM anti-sense primer (5'-GGAA-TCTTAATGTGCTTCTCAGTG-3') were used. The temperature profile was as follows: initial denaturation at 95°C for 10 sec; 40 cycles of denaturation at 95°C for 5 sec, annealing and extension at 60°C for 31 sec; a final cycle at 95°C for 10 sec, 60°C for 1 min and 95°C for 15 sec with continuous fluorescent monitoring. For the real-time IPCR to determine linearity of anti-IgE detection, 10 μg/ml goat anti-human IgE pAb (KPL) was used as capture Ab, and serial dilution of human IgE (Calbiochem, USA) was used as a standard.

RESULTS

Detection of serum IgE specific to Blo t 5 allergen by IPCR

To measure serum titer of Blo t 5-specific IgE detected by

either conventional ELISA or our IPCR the sera from patients #27 and #132 were serially diluted with PBSM, and ELISA and IPCR were performed. Serum from cord blood #2 (CB2) was used as a negative serum control (Fig. 2). ELISA data showed that Blo t 5-specific IgE was detected at a 1:640 dilution of the serum #27, whereas only at a 1:10 dilution of the serum #132 (Fig. 2A), implying that the serum #27 contains about 64-fold higher concentration of IgE for Blo t 5 allergen than the serum #132. GST was used as a negative antigen control, and no specific IgE binding was observed. When Blo t 5-specific IgE in the same serum samples was detected by IPCR, the reporter DNA band on the agarose gel was visible at the 1/10240 dilution of the serum #27, and at the 1/640 dilution of the serum #132 (Fig. 2B). Thus, IPCR seemed about 64-fold more sensitive than ELISA in detecting specific serum IgE without considering the volume of serum samples applied in each assay (50 μl/well for ELISA and 30 μl/well for IPCR), and about 100-fold more sensitive than ELISA with considering the volume of serum samples applied in each assay.

We, then, performed IPCR with more number of serum samples to determine reliability of our approach to measure serum IgE specific to Blo t 5 allergen. Total six serum samples from the patients who are allergic to *B. tropicalis* and three cord blood samples (CB1, CB2 and CB3) were prepared, and IPCR was carried out using 1:100 dilution titer of the serum samples (Fig. 3). The agarose gel analysis of the IPCR products revealed that all patients had serum IgE specific to Blo t 5 but at a different concentration, indicating that IPCR can be utilized successfully to detect Blo t 5-specific serum IgE by using only 300 nl of patient's serum. Expectedly, any significant PCR products were not observed either from the cord blood samples which were used as negative controls, or with GST, a negative antigen control. Among serum samples, the highest Blo t 5-specific serum IgE level was found in serum #27, and serum #132 had the lowest Blo t 5-specific serum IgE.

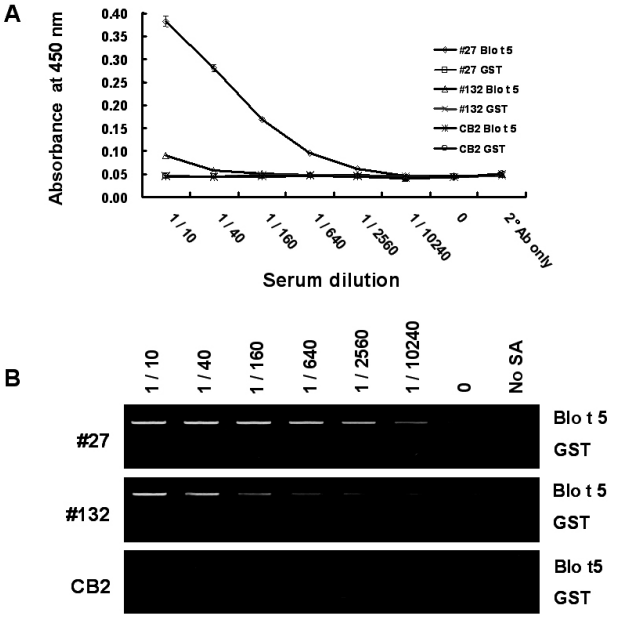

Figure 2. Comparison of the sensitivity of the Blo t 5-specific serum IgE detected by ELISA and the IPCR. Four-fold serial dilutions of the serum samples (patient #27 and #132, and cord blood CB2) were reacted with Blo t 5 mite allergen or GST (negative control antigen) that coated on the microtiter plate for standard ELISA or the heat stable TopYield™ Strips for the IPCR, respectively. In ELISA, streptavidin-conjugated with HRPO and TMB substrate were used to visualize binding signals, and analyzed at OD_{450nm}. Data represent the average of three experiments ± standard deviation. In the IPCR the PCR products were electrophoresed on a 1.5% agarose gel, and the DNA bands were visualized by EtBr staining. Brightness/contrast of the agarose gel picture was adjusted with Power Point software until the background PCR band of the 2° Ab only was just invisible. Details of the procedure are described in *Materials and Methods*.

Real-time IPCR for detecting serum IgE specific for Blo t 5

Instead of analyzing PCR products by using agarose gel electrophoresis, we attempted Real-time IPCR since it enables us to quantify the amount of amplified DNA much more conveniently over a wide concentration range. To confirm the linearity of PCR amplification proportional to IgE concentration, the control experiments were performed using the TopYield™ Strips coated with anti-human IgE pAb and a

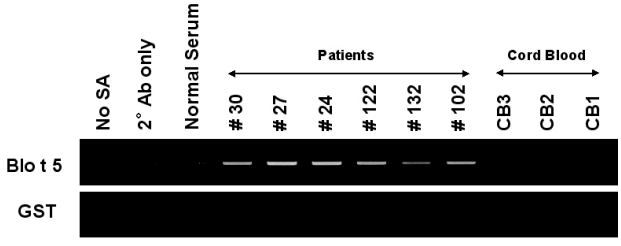

Figure 3. Identification of serum IgE specific for Blo t 5 allergen by the IPCR. Nine different sera in a 1:100 dilution were used in IPCR to identify Blo t 5-specific serum IgE by the IPCR using the TopYield™ Strips coated with Blo t 5 or GST. The resulting PCR products were electrophoresed on a 1.5% agarose gel, and the DNA bands were visualized by EtBr staining. Brightness/contrast of the agarose gel picture was adjusted with Power Point software until the background PCR band of the 2° Ab only was just invisible.

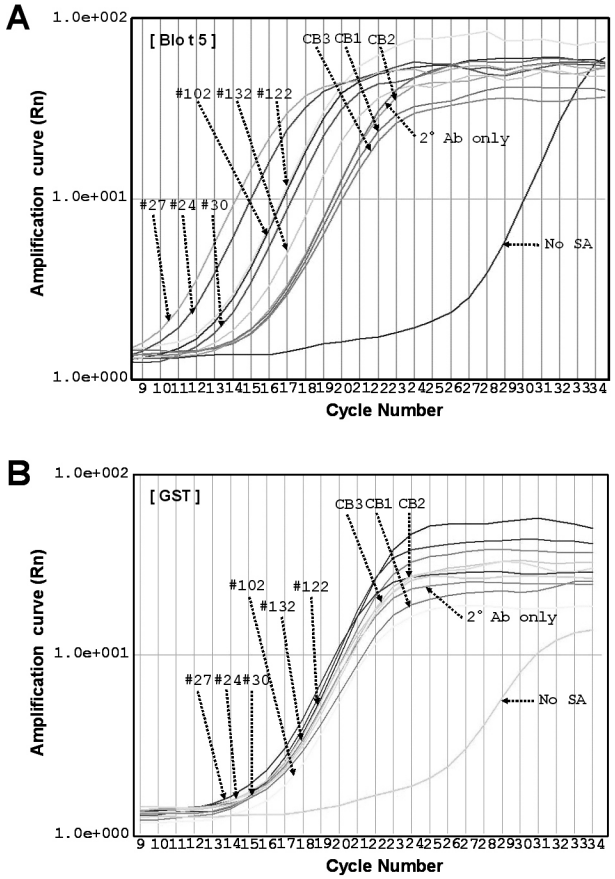

Figure 4. Amplification curves obtained by the real-time IPCR to determine the amplification of reporter DNA proportional to specific serum IgE concentration. The TopYield™ Strips were coated with Blo t 5 (A) or GST (B), and nine different sera at a 1:100 dilution were used for the real-time IPCR as described in *Material and Methods*.

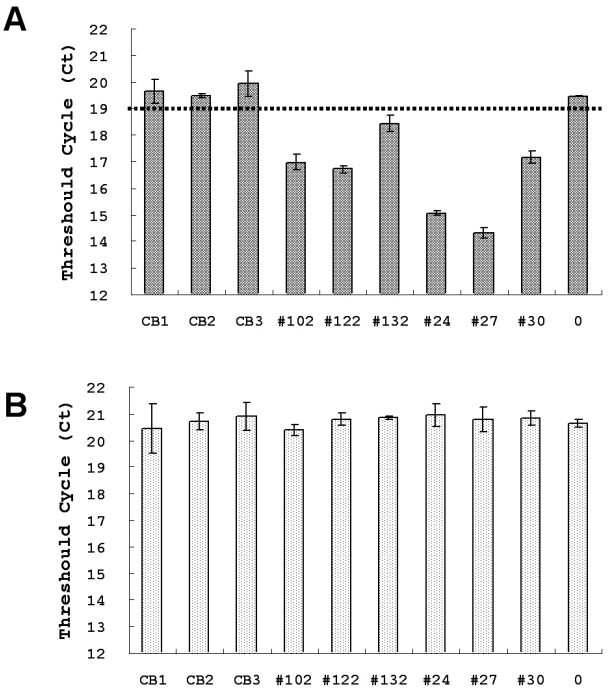

Figure 5. Average and standard deviation of the Ct values obtained from the triplicate real-time IPCR. Real-time IPCR in Fig. 4 was carried out three times using the TopYield™ Strips that coated with Blo t 5 (A) or GST (B), and graphs were plotted by using the average± standard deviations that calculated the Ct corresponding to a fluorescence threshold at the exponential phase of the amplification curves. Cts which are statistically below those of three cord blood samples (CB) are indicated by a dotted line.

pre-determined concentration of human IgE ranging from 12 ng/ml to 0. Linear PCR amplification curves were observed from 12 ng/ml to 187.5 pg/ml of IgE, and the detection limit of IgE was approximately 5.7 pg or 1.8×10^7 IgE molecules in our experimental setup (data not shown). As in Fig. 3, nine different sera at 1:100 dilution were analyzed in the real-time IPCR to determine specific IgE for Blo t5 (Fig. 4A) and GST (Fig. 4B). The amplification curves shown in Fig. 4 were exactly correlated with the amount of PCR products in Fig. 3, yet real-time PCR was far more convenient in comparing the allergen-specific IgE levels between serum samples.

Thereafter, the identical real-time IPCR was performed in triplicate to confirm the reproducibility of the experiments, and average and standard deviations were calculated with the threshold cycle (Ct) corresponding to a fluorescence threshold at the exponential phase of the amplification curves (Fig. 5A). Well-to-well standard deviation was between 0.08 to 0.55 Ct, and all six Blo t 5-positive sera exhibited Cts below those of cord blood samples (CB), indicated by a dotted line, in a statistically significant manner, demonstrating the feasibility of the real-time IPCR in detecting specific serum IgE. As in the Fig. 4B no significant PCR products were obtained when GST was used to capture serum IgE, which are shown as no significant variation in Ct values among serum samples (Fig. 5B).

Detection of serum IgE reactivity with other mite allergens by IPCR

To determine the cross-reactivity of serum IgE to other mite allergens, IPCR was performed using a recombinant Der p 1 and Der p 2 allergen (Fig. 6). Serum samples diluted at 1:100 were also utilized. The agarose gel analysis of the IPCR

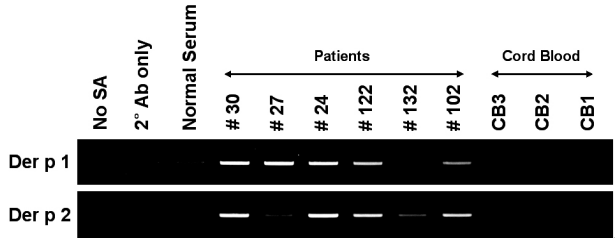

Figure 6. Identification of the serum IgE reactivity to Der p 1 or Der p 2 mite allergens by the IPCR. The sera from nine different patients were diluted at 1:100, and mite allergen-specific IgE in the samples were determined by the IPCR using the TopYield™ Strips coated with Der p 1 or Der p 2. The resulting PCR products were electrophoresed on a 1.5% agarose gel, and the DNA bands were visualized by EtBr staining. Brightness/contrast of the agarose gel pictures was adjusted until the background PCR band of the 2° Ab only (Der p 1) or normal serum (Der p 2) disappeared using Power Point software.

products showed that five out of six samples (#30, #27, #24, #122 and #102) contain IgE that cross-reacts with Der p 1. Similarly, five out of six sera (#30, #24, #122, #132 and #102) also have Der p 2-specific serum IgE. It is interesting to note that the patient #27 seemed to have serum IgE specific for Blo t 5 and Der p 1, but not for Der p 2. To confirm the absence of Der p 2-specific IgE in the serum #27, the identical IPCR was carried out using 10-fold higher concentration of the serum, and no PCR product was obtained. In the case of the patient #132, serum IgE specific for Der p 1 was not detected.

DISCUSSION

One of disadvantages of the IgE immunoassays is a low detection limit that may produce discrepant results to the skin testing, and they usually require large quantities of serum samples to carry out. Since it is certain that improvement on the sensitivity of the immunoassay contributes to a more accurate diagnosis of allergic diseases, the ultra-sensitive IPCR method was applied to determine serum IgE against Blo t 5 allergen as a model.

As described by Sano et al. the IPCR allows the highly specific and sensitive detection of analyte by taking advantage of combining the antigen-antibody interaction with the amplification power of the PCR (14). In a standard IPCR protocol, target-specific antibody is coupled to a reporter DNA either by direct conjugation or *via* streptavidin-biotin interaction (15,16), followed by PCR amplification of the reporter DNA of which copy number is directly proportional to specific antibody bound to a target molecule. Similarly, we utilized streptavidin to link biotinylated reporter DNA and biotinylated anti-human IgE antibody as in previous reports (17). Various reporter DNA moieties originated from different sources including glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (30), pUC19 (31), pBluescript (22,32), λ phage DNA (33), α-hydroxynitrile lyase of the cassava plant (34), oligonucleotides (27) or the *E. coli beta-glucuronidase* (GUS) gene (35) had been successfully utilized without any functional differences. In this study, reporter DNA was designed from rice starch synthase cDNA, and milk solution was used as a blocking agent to improve signal-to-noise ratios (27). Additionally, we had used a normal human serum which is commercially available to dilute patient's serum samples as previously reported by Lind and Kubista (16), but found that the normal serum we used contains a low level of Der p 2-specific IgE, suggesting that careful attention must be paid in choosing a normal human serum for IPCR.

In the present report we demonstrate the feasibility of using IPCR for the sensitive detection of Blo t 5-specific IgE antibodies in human sera substantiated by perfect concordance between the IPCR and ELISA. It had been proposed that a 1000- to 10,000-fold increase in detection sensitivity can be obtained by IPCR compared to ELISA (14), or even 100,000- to 1,000,000- fold lower concentrations of the target molecules can be detected by IPCR than those detected by western blot and ELISA (36). In our experimental setup, however, we were able to achieve only 100-fold higher sensitivity than ELISA similar to the report by Lind and Kubista (16). The exact cause of a lower sensitivity in our system is not known since PCR amplification in the IPCR can be affected by several factors including optimal concentration of reagents, presence of non-specific background noise and affinity of specific antibodies. From the experiment using human IgE standards, we were able to determine that our assay limit of IgE was about 187.5 pg/ml which is much higher than that of previous reports demonstrating successful detection of 100 ~ 1 fg/ml of target molecules (22,31,36). Most recently, however, the carefully scrutinized study by Niemeyer et al. (37) revealed that up to 10 pg/ml of IL-6 can be measured by the IPCR with antibody-streptavidin-reporter DNA conjugate, whereas 100 fg/ml of the molecule can be assayed by IPCR using a direct antibody-DNA conjugate. Indeed, we were also able to achieve the reproducible detection of up to 12 pg/ml IgE by using a 10-fold lower concentration of streptavidin (10 ng/ml) than that used in the present study (data now shown), and we assume that preparation of anti-human IgE secondary

antibody directly conjugated with reporter DNA would be preferred for detection of lower IgE concentration in serum.

The significance of our study is that 0.3 μl of serum is enough to detect allergen-specific serum IgE, whereas current serum IgE immunoassays such as ELISA, the RAST, and the CAP system require much larger quantities of serum samples. Allergy has become more prevalent recently and it has been estimated that the frequency of food allergies in infants is approximately 2～5% (38). Considering that only limited amounts of serum can be obtained from infants, application of IPCR in determining allergen-specific IgE can be a valuable tool since only 1 ml of serum is good enough to screen for over 300 of the most common allergens in a throughput manner. This is especially notable when taking advantage of real-time IPCR which has proved to be very useful in the assay by providing rapid and reproducible quantification of reporter DNA, and that analysis can be completed considerably faster than by conventional PCR. None of the assays currently used in the diagnosis of allergies has the high-throughput to screen for allergens, and the development of sophisticated technologies including microarrays are in progress to overcome present limitations (39). Although we studied a small number of patient sera against a very limited number of mite allergens, our results clearly show that IPCR can be applied in diagnosing specific serum IgE in a dependable manner. Moreover, we believe that automation of the real-time IPCR assay format and calibration of the results in kIU/L would provide allergy clinicians with not only quantitative, but also multi-analytical measurements that will aid more accurate diagnosis of allergies.

ACKNOWLEDGEMENT

We'd like to thank Dr. Hong, Cheon-soo (School of Medicine, Yonsei University, South Korea) for his kind and thoughtful comments in preparing this manuscript.

REFERENCES

1. Sly RM: Changing prevalence of allergic rhinitis and asthma. Anm Allergy Asthma Immunol 82;233-248, 1999
2. Nelson HS: Variables in allergy skin testing. Allergy Proc 15;265-268, 1994
3. McCann WA, Ownby DR: The reproducibility of the allergy skin test scoring and interpretation by board-certified/board-eligible allergists. Ann Allergy Asthma Immunol 89;368-371, 2002
4. Dolen WK: Manual of Clinical Laboratory Immunology. In: Rose NR, Hamilton RG, Detrick B eds.: p883-890, Washington DC ASM Press, 2002
5. Ekins R: Merits and Disadvantages of Different Labels and Methods of Immunoassay. MTP Press, 1981.
6. Wide L, Bennich H, Johansson SGO: Diagnosis of allergy by an in-vitro test of allergen antibodies. Lancet 2;1105-1107, 1967
7. Axen R, Drevin H, Kober A, Yman L: A new Laboratory Diagnostic System Applied to Allergy Testing. In: Johansson SGO eds.: IgE Antibodies and the Pharmacia CAP System in Allergy Diagnosis. p3-5, Pharmacia Publications, 1988
8. Ruan KH, Kulmacz RJ, Wilson A, Wu KK: Highly sensitive fluorimetric enzyme immunoassay for prostaglandin H synthase solubilized from cultured cells. J Immunol Methods 162;23-30, 1993
9. Stanley CJ, Johannsson A, Self CH: Enzyme amplification can enhance both the speed and the sensitivity of immunoassays. J Immunol Methods 83;89-95, 1985
10. Johannsson A, Ellis DH, Bates DL, Plumb AM, Stanley CJ: Enzyme amplification for immunoassays: detection limit of one hundredth of an attomole. J Immunol Methods 87;7-11, 1986
11. Williams PB, Barnes JH, Szeinbach SL, Sullivan TJ: Analytic precision and accuracy of commercial immunoassays for specific IgE: establishing a standard. J Allergy Clin Immunol 105;1221-1230, 2000
12. Williams PB, Dolen WK, Koepke JW, Selner JC: Comparison of skin testing and three in vitro assays for specific IgE in the clinical evaluation of immediate hypersensitivity. Ann Allergy 68;35-45, 1992
13. van der Zee JS, de Groot H, van Swieten P, Jansen HM, Aalberse RC: Discrepancies between the skin test and IgE antibody assays: study of mediator release, complement activation in vitro, and occurrence of allergen-specific IgG. J Allergy Clin Immunol 82;270-281, 1988
14. Sano T, Smith CL, Cantor CR: Immuno-PCR: very sensitive antigen detection by means of specific Ab-DNA conjugates. Science 258;120-122, 1992
15. Hendrickson ER, Truby TM, Joerger RD, Majarian WR, Ebersole RC: High sensitivity multianalyte immunoassay using covalent DNA-labeled antibodies and polymerase chain reaction. Nucl Acids Res 23;522-529, 1995
16. Lind K, Kubista M: Development and evaluation of three real-time immuno-PCR assemblages for quantification of PSA. J Immunol Methods 304;107-116, 2005
17. Zhou H, Fisher RJ, Papas TS: Universal immuno-PCR for ultra-sensitive target protein detection. Nucleic Acids Res 21;6038-6039, 1993
18. Adler M, Schulz S, Fischer R, Niemeyer CM: Detection of Rotavirus from stool samples using a standardized immuno-PCR ("Imperacer") method with end-point and real-time detection. Biochem Biophys Res Commun 333; 1289-1294, 2005
19. Sims PW, Vasser M, Wong WL, Williams PM, Meng YG: Immunopolymerase chain reaction using real-time polymerase chain reaction for detection. Anal Biochem 281;230-

232, 2000
20. Sanna PP, Weiss F, Samson ME, Bloom FE, Pich EM: Rapid induction of tumor necrosis factor alpha in the cerebrospinal fluid after intracerebroventricular injection of lipopolysaccharide revealed by a sensitive capture immuno-PCR assay. Proc Natl Acad Sci USA 92;272-275, 1995
21. Saito K, Kobayashi D, Sasaki M, Araake H, Kida T, Yagihashi A, Yajima T, Kameshima H, Watanabe N: Detection of human serum tumor necrosis factor-alpha in healthy donors, using a highly sensitive immuno-PCR assay. Clin Chem 45;665-669, 1999
22. Furuya D, Yagihashi A, Yajima T, Kobayashi D, Orita K, Kurimoto M, Watanabe N: An immuno-polymerase chain reaction assay for human interleukin-18. J Immunol Methods 238;173-180, 2000
23. Maia M, Takahashi H, Adler K, Garlick RK, Wands JR: Development of a two-site immuno-PCR assay for hepatitis B surface antigen. J Virol Methods 52;273-286, 1995
24. Mweene AS, Ito T, Okazaki K, One E, Shimizu Y, Kida H: Development of immuno-PCR for diagnosis of bovine herpesvirus 1 infection. J Clin Microbiol 34;748-750, 1996
25. Kakizaki E, Yoshida T, Kawakami H, Oseto M, Sakai T, Sakai M: Detection of bacterial antigens using immuno-PCR. Lett Appl Microbiol 23;101-103, 1996
26. Wu HC, Huang YL, Lai SC, Huang YY, Shaio MF: Detection of Clostridium botulinum neurotoxin type A using immuno-PCR. Lett Appl Microbiol 32;321-325, 2001
27. McKie A, Samuel D, Cohen B, Saunders NA: Development of a quantitative immuno-PCR assay and its use to detect mumps-specific IgG in serum. J Immunol Methods 261;167-175, 2002
28. Kuo IC, Cheong N, Trakultivakorn M, Lee BW, Chua KY: An extensive study of human IgE crossreactivity of Blo t 5 and Der p 5. J Allergy Clin Immunol 111;603-609, 2003
29. Ren J, Chen Z, Zhou SJ, Zhang XY, Pan BR, Fan DM: Detection of circulating gastric carcinoma-associated antigen MG7-Ag in human sera using an established single determinant immuno-polymerase chain reaction technique. Am Cancer Soc 88;280-286, 1995
30. Sperl J, Paliwal V, Ramabhadran R, Nowak B, Askenase PW: Soluble T cell receptors: detection and quantitative assay in fluid phase via ELISA or immuno-PCR. J Immunol Methods 186;181-194, 1995
31. Niemeyer CM, Adler M, Blohm D: Fluorometric polymerase chain reaction (pcr) enzyme-linked immunosorbent assay for quantification of immuno-pcr products in microplates. Anal Biochem 246;140-145, 1997
32. Sugawara K, Kobayashi D, Saito K, Furuya D, Araake H, Yagihashi A, Yajima T, Hosoda K, Kamimura T, Watanabe N: A highly sensitive immuno-polymerase chain reaction assay for human angiotensinogen using the identical first and second polyclonal antibodies. Clinica Chimica Acta 299;45-54, 2000
33. Chang TC, Huang SH: A modified immuno-polymerase chain reaction for the detection of b-glucuronidase from *Escherichia coli*. J Immunol Methods 208;35-42, 1997
34. Case MC, Burt AD, Hughes J, Palmer JM, Collier JD, Bassendine MF, Yeaman SJ, Hughes MA, Major GN: Enhanced ultrasensitive detection of structurally divere antigens using a single immuno-PCR assay protocol. J Immunol Methods 223;93-106, 1999
35. Liang H, Cordova SE, Kieft TL, Rogelj S: A highly sensitive immuno-PCR assay for detecting Group A Streptococcus. J Immunol Methods 279;101-110, 2003
36. Barletta JM, Edelman DC, Highsmith WE, Constantine NT: Detection of ultra-low levels of pathologic prion protein in scrapie infected hamster brain homogenates using real-time immuno-PCR. J Virol Methods 127;154-164, 2005
37. Niemeyer CM, Adler M, Wacker R: Detecting antigens by quantitative immuno-PCR. Nature Protocols 2;1918-1930, 2007
38. Ring J, Brochow K, Berendt H: Adverse reactions to foods. J Chromatogr 36;3-10, 2001
39. Bacarese-Hamilton T, Mezzasoma L, Ingham C, Ardizzoni A, Rossi R, Bistoni F, Crisanti A: Detection of Allergen-specific IgE on Microarrays by Use of Signal Amplification Techniques. Clinical Chemistry 48;1367-1370, 2002