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Application of immuno-PCR assay for the detection of serum IgE specific to Bermuda allergen

Samina Rahmatpour1,7¥, Amjad Hayat Khan2¥, Rasoul Nasiri Kalmarzi3, Masoumeh Rajabibazl4,5, Gholamreza Tavoosidana6, Elahe Motevaseli6, Nosratollah Zarghami1, Esmaeil Sadroddiny7*

1. Department of Clinical Biochemistry and Laboratory Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.
2. Department of Medical Biotechnology, School of Advanced Technologies in Medicine, International Campus, Tehran University of Medical Sciences, Tehran, Iran.
3. Cellular and Molecular Research Center, Kurdistan University of Medical Sciences, Sanandaj, Iran.
4. Department of Clinical Biochemistry, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
5. School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
6. Department of Molecular Medicine, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran.
7. Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran

¥ equally contributed

*Corresponding Author:

Dr. Esmaeil Sadroddiny, PhD, Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran
Email: sadroddiny@sina.tums.ac.ir
Abstract

*In vivo* and *in vitro* tests are the two major ways of identifying the triggering allergens in sensitized individuals with allergic symptoms. Both methods are equally significant in terms of sensitivity and specificity. However, in certain circumstances, *in vitro* methods are highly preferred because they circumvent the use of sensitizing drugs in patients. In current study, we described a highly sensitive immuno-PCR (iPCR) assay for serum IgE specific to Bermuda allergens. Using oligonucleotide-labelled antibody, we used iPCR for the sensitive detection of serum IgE. The nucleotide sequence was amplified using conventional PCR and the bands were visualized on 2.5% agarose gel. Results demonstrated a 100-fold enhancement in sensitivity of iPCR over commercially available enzyme-linked immunosorbent assay (ELISA) kit. Our iPCR method was highly sensitive for Bermuda-specific serum IgE and could be beneficial in allergy clinics.

**Keywords**: immuno-PCR, detection of serum IgE, diagnosing allergy, *in vitro* IgE tests, detecting type I allergy
1 Introduction

Immunoglobulin E (IgE) is the least abundant and fifth class of immunoglobulins, providing first line of defense against parasitic infestation. Besides, IgE is one of the major mediators of immediate hypersensitivity reactions that underlie atopic conditions such as, urticaria, seasonal allergy, asthma, and anaphylaxis [1, 2]. The growing prevalence of allergies particularly, asthma [3] has motivated the research community to understand the structure as well as the interaction of IgE with other proteins. The interaction between IgE and effector cells takes place through a network of receptor proteins: Fc\(\epsilon\)RI (high-affinity receptors) and Fc\(\epsilon\)RII (low-affinity receptors) [4, 5]. Two major tests: skin testing and serum assays for allergen-specific IgE have been employed in allergy diagnostic clinics. In former approach, a small amount of diluted allergen is delivered to the body through pricking or scratching the skin, or intradermal injection, and the skin is used as a mirror of cells present in nose or lungs [6, 7]. This method is rapid, sensitive, inexpensive, and the best available option employed in allergy clinics. Unfortunately, due to associated adverse events skin testing is impractical to perform in patients with a risk of anaphylaxis, who cannot discontinue interfering medications, or suffering from skin diseases. In contrast, in vitro tests are safe and could be used as substitutes [8, 9]. In addition, in vitro allergy testing also provides the opportunity to monitor the clinical efficacy of commercially available anti-IgE antibody (omalizumab) in patients [10, 11].

During the past decades, our understanding about the molecular structure of allergens has increased dramatically. Several allergens have been identified, cloned, and expressed as recombinant proteins [12, 13]. Numerous antibodies have been produced for therapeutic applications and research [14, 15] that could be used for the development of innovative detection tools for serum/urine biomarkers. Since long, enzyme-linked immunosorbent assay (ELISA) has been used in clinical settings for diagnosis, but sensitivity limits restricted its use in modern diagnostic laboratories. For increasing the sensitivity of protein detection, immuno-PCR (iPCR) has been exploited [16-18]. The amplification power of PCR has been shown to increase the sensitivity range of iPCR from 100-10,000-fold compared to the analogous ELISA [19]. So far, iPCR has been used for the detection of cancer biomarkers [17, 20], viral antigens [21], antibodies in infection [22], and serum IgE specific to mite allergens [23].

Bermuda grass (Cynodon dactylon) pollen (BGP) is one of the most common causes of airway allergic disease in subtropical and temperate regions of many countries, containing 12 allergenic
proteins [24]. Of which, very few have been identified and characterized [25]. Cyn d 1 is a major allergen and most abundant protein in BGP, comprising 15% of the whole pollen extract. Studies have revealed that more than 96% of individuals allergic to BGP were hypersensitive to cyn d 1 [25-27]. This study aimed to develop a sensitive and specific iPCR assay for the detection of serum IgE specific to BGP.

2 Materials and methods

2.1 Allergen immobilization

A sterile solution of standardized grass pollen extracts from BGP (GREER® Hollister Stier, Co) was coated in 96-well ELISA plate. The allergenic extract with a concentration of 10,000 BAU/mL-225 µg/mL was serially diluted in PBS (pH 7.4) to 5000, 2000, 1000, 100, 10, 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} BAU/mL. 40 µL of allergen from each dilution was coated by incubating the wells at 4°C overnight. Well were washed four times with TBS (pH 7.6). Non-adsorbed sites were blocked with 200 µL of blocking buffer containing, TBS, 4.5% skimmed milk, and 5 mM EDTA. After blocking at room temperature for one hour, wells were washed five times with TBS-TE (0.05% tween 20 and 5 mM EDTA in TBS).

2.2 Addition of serum IgE and biotin-detection antibody

Serum samples of patients, sensitized to BGP and mite allergens, were collected from diagnostic laboratory (Cellular and Molecular Research Center, Kurdistan University of Medical Sciences). In each sample the concentration of IgE was: 180, 245, 315, 356 IU/mL, which was already determined with total IgE ELISA kit (PISHTAZ TEB DIAGNOSTICS, cat # PT-IgE-96). Protocol for iPCR was optimized with serum containing IgE, 356 IU/mL-855 ng/mL. Serum was serially diluted and in each dilution the concentration of IgE was 356×10^{-1}, 356×10^{-2}, 356×10^{-3}, 356×10^{-4}, 356×10^{-5}, 356×10^{-6}, 356×10^{-7}, 356×10^{-8}, 356×10^{-9}, 356×10^{-10} IU/mL. 40 µL from each dilution was added to the coated wells. Following incubation at 37°C for 30 minutes, wells were washed five times with TBS-TE. While for determining the specificity of the assay, serum containing IgE specific to mite allergens was added to BGP-coated wells. Biotin-anti-human IgE 0.5 mg/mL (BioLegend, Cat#325503) was diluted in PBS (pH 7.4) to a final concentration of 1 µg/mL and 40 µL from this dilution was added to the wells. After incubation at 37°C for 30 minutes, wells were washed five times with TBS-TE.
2.3 Conjugation of streptavidin with biotinylated-DNA

An 80bp biotinylated DNA sequence (CGCATCGCCCTTGGACTACGACTGACGAACGCCTGACTGATCGCTTCTGATCGTGTC TAAAGTCCGTACCTTTGATTCCC), forward primer (5’-CATCGCCCTTGGACTACGA-3’), and reverse primer (5’-GGGAATCAAGGTAACGGACTTTAG-3’) were synthesized. Conjugates of streptavidin (STV) and biotinylated-DNA were prepared by mixing 2 pmol of STV and 1 pmol of biotinylated DNA in buffer containing, 0.01 M Tris-HCl (pH 7.3) and 0.005 M EDTA at room temperature for 30 minutes. 30 µL of conjugates 1:20 dilution in TBS was added to wells and incubated at room temperature for 30 minutes. Washing was done five times with TBS-TE followed by five time washing with TBS only. Unbound DNA was washed off by incubating the wells with TBS for one hour. The buffer was removed by patting the wells on adsorbent surface.

2.4 Detachment of DNA for PCR

The wells were not compatible with the holes in thermocycler therefore, DNA was detached from antigen-antibody complex. For this purpose, PCR master mix containing, 12.5 µL of 2X Taq DNA Polymerase Master Mix Red (AMPLIQON: Cat. No:180301) and 10.5 µL PCR grade water was added to each well. The wells were sealed with parafilm and incubated at 94°C for 5 minutes. PCR master mix along with detached DNA was transferred to marked PCR tubes and 1 µL from each forward and reverse primers (10 µM) was added. Temperature profile for 30 cycles of PCR was set: initial denaturing (95°C, 4 minutes), denaturation (95°C, 30 sec), annealing (56°C, 40 sec), extension (72°C, 1 min), and final extension (72°C, 5 min). Two PCR tubes: one containing master mix, primers, and 2.5 µL reporter DNA while other containing distilled water instead of reported DNA were also included as positive and negative controls. Finally, PCR products were loaded on 2.5% agarose gel, electrophoresed, and visualized under UV light.

3 Results

3.1 Development of iPCR protocol

By coating BGP in 96-well ELISA plate, a highly sensitive and specific iPCR assay for the detection of serum IgE was developed. Non-adsorbed sites were blocked with blocking buffer and serum from BGP-sensitized individual was added to the wells. Following incubation and
washing, biotinylated anti-human IgE antibody and STV-biotinylated DNA conjugates were added. Unbound DNA was eliminated by washing the wells several times. Antigen-antibody complex formed during the process interferes with PCR. Therefore, DNA was detached from biotin-streptavidin conjugates by incubating the wells with PCR master mix without primers at 94 °C for 5 minutes. PCR master mix containing detached DNA was transferred to PCR tubes, and both forward and reverse primers were added. PCR was carried out, the product was loaded on 2.5% agarose gel. After electrophoresis, bands were visualized under UV light and photographed (Figure 1).

3.2 Determining the sensitivity of iPCR
First of all, we determined the least amount of allergen that could be used for coating. For this purpose, the antigen was serially diluted and each dilution was coated in ELISA plate wells. Serum IgE (0.03 IU/mL) was added to each well. After performing all succeeding steps of iPCR, the PCR product from each well was loaded on 2.5% agarose gel. The density of bands diminished along with decrease in the concentration of allergen in each dilution. A faint band for dilution containing 0.01 BAU/mL of allergen was visualized. However, no band was observed for dilutions containing allergen less than 0.01 BAU/mL (Figure 2). For optimization of iPCR protocol for serum IgE, 1500 BAU/mL of BGP was used for coated.
In order to determine the sensitivity limit of iPCR for BGP-specific serum IgE, all dilutions of serum containing different concentrations of IgE were added to the wells. All the steps of optimized iPCR protocol were followed. For each dilution, DNA bands on 2.5% agarose gel were observed. The density of bands slipped to diminish along with decrease in concentrations of IgE in each dilution. A faint band for serum dilution containing IgE 0.03 IU/mL was seen (Figure 3). No band was visible for serum dilutions containing IgE less than 0.03 IU/mL and this was the sensitivity limit of our iPCR assay. Moreover, no bands were observed in control samples that were obtained from patients allergic to mite allergens. Absence of bands in control lanes confirmed the specificity of the assay for BGP-specific serum IgE. A band for positive control was also observed while no band was seen in negative control lane.

4 Discussion
Allergy testing is an important component of the evaluation and management of allergic diseases, because clinical history alone is not sufficient for identifying the specific allergen
causing the symptoms of allergy. Accurate diagnosis of triggering or causative allergen is essential for appropriate advice of avoidance and environmental control measures. The two most commonly used methods of confirming the allergen sensitization are skin testing (in vivo) and measurement of allergen-specific serum IgE (in vitro) [28]. In vivo testing indirectly measures the reactivity of IgE bearing cutaneous mast cells that are present under the sub-epithelial layer of skin, respiratory, nasociliary, and gastrointestinal tracts. Among all these areas, skin is the most suitable and easily accessible area for prick/puncture procedures. Skin testing is rapid, reliable, and inexpensive method for diagnosing IgE-mediated allergy in patients with rhinoconjunctivitis, asthma, urticaria, atopic eczema, and suspected food and drug allergy [7]. However, this method is not universal and cannot be used in pregnant mothers, unstable medical conditions like patients with unstable asthma or reduced lung function, and recent stroke or cardiac events [29]. Moreover, skin reactions can be influenced by certain medications and dermatologic conditions. In contrast, in vitro tests are safe, more specific, and not influenced by medications and dermatologic conditions.

Several in vitro methods have been developed for determining IgE levels in serum [30-32]. Currently, commercially available ELISA kit (PISHTAZ TEB DIAGNOSTICS, cat # PT-IgE-9s6) is in clinical practice for detecting total IgE in serum. However, lower sensitivity limits constrain the use of conventional ELISA in allergy-immunology clinics. Reliable, sensitive, and specific methods are needed to improve the diagnostic accuracy of allergen-specific serum IgE detection. In this regard, a highly powerful and sensitive detection tool, iPCR combining the versatility of ELISA with the amplification power of PCR is considered more beneficial. The enormous exponential amplification power of PCR increases the sensitivity of iPCR to at least 100-10,000-fold compared with analogous ELISA. It has been used to detect several biomarkers including, toxins, cytokines, hormones, and microbial antigens and antibodies [17, 19, 33].

Here we described the adaptation of iPCR for the detection of BGP-specific serum IgE. The sensitivity limit of iPCR was 0.03 IU/mL=0.072 ng/mL, 100-fold more than the commercially available ELISA (1 IU/mL = 2.4 ng/mL). While the sensitivity of newly developed ELISA was 9.38 ng/mL that that has been developed for monitoring serum free IgE levels during omalizumab therapy [34]. Nevertheless, compared to the results of Lee et al [23] in which iPCR was used for the detection of serum IgE specific to mite allergens and other studies claiming 1000-fold sensitivity improvement over ELISA [35], the sensitivity of our assay was less. Loss
in sensitivity could be attributed to the use of ELISA plate for coating allergens and conventional PCR for signal generation. Because wells of ELISA plate are not compatible with the holes in thermocycler therefore, DNA must be transferred from wells to PCR tubes, and this may influence the sensitivity of the assay. Although conventional PCR is economical, easily available in diagnostic laboratories, and possibly be used for DNA amplification in iPCR [36], but somehow it is laborious and time consuming [37]. On the other hand, real-time PCR is expensive with respect to accessory kits and often restricted to special laboratories, however, its sensitivity, dynamic range, and precision is more than the conventional PCR and gives good results in a very short time [37]. Due to these advantages, majority of the recently developed iPCR assays have focused on real-time PCR for signal generation [35, 38]. No bands on gel were observed for sera containing IgE specific to mite allergens, confirming the specificity of the assay. Hence, this assay could be employed for the inexpensive and sensitive detection of BGP-specific serum IgE in clinical settings. However, for enhancing the sensitivity it is highly recommended to use real-time PCR for signal generation.

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References


**Figure 1.** Schematic presentation of iPCR protocol for detection of serum IgE in Bermuda-sensitized individuals, B: Biotin, STV: Streptavidin.

**Figure 2.** The density of bands from lane 2 (10,000 BAU/mL) to lane 10 (0.01 BAU/mL) decrease along with decrease in concentration of BGP in each consecutive dilution. No bands from lane 11 to 14 for further dilutions of BGP were observed. Lane 15 to 17: empty, lane 18: negative control without template DNA, Lane 19: positive control, and lane 1: ladder.

**Figure 3.** The density of bands for Bermuda-specific serum IgE from lane 2 to 8 decrease with decrease in concentration of IgE in each dilution. Lane 8: (0.03 IU/mL), the least amount of IgE detected by iPCR. Lane 9 to 14: no visible band for further serum dilutions. Lane 15 and 17: non-dilute serum IgE from individuals allergic to dust mites, lane 18: negative control without template DNA, lane 19: positive control, and lane 1: ladder.
- A sensitive iPCR for serum IgE specific to Bermuda allergen is presented
- The assay demonstrates high specificity and sensitivity for serum IgE
- This iPCR is 100-fold sensitive for serum IgE compared to conventional ELISA kit