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

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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.

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: FcεRI (high-affinity receptors) and FcεRII (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

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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 to 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 1 h, 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 356,178, 71.2, 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 min, 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 min, wells were washed five times with TBS-TE.

2.3. Conjugation of streptavidin with biotinylated-DNA

An 80bp biotinylated DNA sequence (CGCATCGCCCTTGGACTACGACTGACGAACGCCTGACTGATCGCTTCGTGTCGTGTCTAAAGTCCGTTACCTTGATTCCC), 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 min 30 μL of conjugates 1:20 dilution in TBS was added to wells and incubated at room temperature for 30 min. 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 1 h. 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 min. 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 min), denaturation (95 °C, 30 s), annealing (56 °C, 40 s), 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 min. 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 (Fig. 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