Original Article

Development of PCR-Based Method for Rapid Detection of Abrin Gene

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Abstract

Abrin, known as a ribosome inactivating protein (RIP), is a high cytotoxic plant protein. The high lethality, low cost, and easy access to this plant and its seeds have led to this toxin to be used in crimes and terrorist acts. Since, obtaining purified toxins requires advanced laboratory equipment and complex procedures, it seems that the perpetrators of such crimes use crude extracts. As a result, it was hypothesized that remaining the specific toxin genes in these extracts can provide the advantage of using PCR assay to identify abrin gene which refers to the existence of its toxin. We used a new rapid molecular method for the detection of the abrin gene by PCR. In this regard, specific primers were designed and the required DNA was extracted from Rosary pie samples using cetyltrimethylammonium bromide-polyvinylpyrrolidone (CTAB-PVP) method and PCR protocol was performed using specific primers. Then, assay's sensitivity was analyzed using serial dilution method. The results of this study revealed that designed and selected primers sequence for toxin's gene function as specific. The desired product size was obtained and sequencing of PCR products showed up to 90% similarity with known sequence for each molecule. According to these results, the developed rapid molecular method for detection of abrin toxin gene can be considered as a sensitive and low-cost detection method for this toxin gene in cases of suspection to bioterrorism event.

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Introduction

Abrin is a plant-originated toxin derived from rosary peas (*Abrus precatorius* L.). It is listed in the category B agents of potential bioterrorism risk by the 'Centers for Disease Control and Prevention Moran' [1]. It is also classified as Type 2 ribosome-inactivating proteins. Its extreme toxicity, easy production process, and availability from natural sources make it a potential biological warfare agent or terrorist weapon [2, 3]. The mechanism of toxicity action of abrin is identical to that of ricin, but the toxicity of abrin in mice is 75 times more than ricin. Ricin and abrin also resemble in diagnosis, treatment, clinical features, protection, prophylaxis, etc. [4, 5]. Abrin toxin is a heterodimeric glycoprotein that consists of two polypeptides, the A-chain (approximately 30 kDa) and B-chain (approximately 32 kDa) polypeptide are linked together by a disulfide bond [6].

B-chain is a lectin responsible for binding to the cell surface receptors such as glycoproteins and glycolipids that contain β-1,4-linked galactose residues, it facilitates the toxin internalization into the cells while A-chain acts on the ribosomes by its N-glycosidase activity. It irreversibly removes an adenine from the position 4,324 of the 28S rRNA in the 60S ribosomal subunit.

The depurinated rRNA is then unable to bind protein elongation factor-2. Therefore, toxication leads to protein synthesis failure and eventually cell death [7, 8]. Consequently, rapid and reliable detection of this toxin is very important. In this study, we tried to develop a PCR-based detection method for abrin toxin's gene.

Materials and Methods

Abrin seeds sample

Seeds were obtained from seed traders or herbal remedies sellers. To produce an actual case sample, we tried to adapt the extraction process from "terrorist cookbook" previously used and referenced by Eva Felder *et al*., [9]. The 3% hypochlorite solution was used for decontamination of all workplace, labware, and containers.

DNA extraction

For DNA preparation from Abrus precatorius seeds, two seeds were placed in liquid nitrogen temperature for 3 minutes and then their hard shell was crashed in a mortar-pestle described aboveand gently grinded. After crushing the seeds, the obtained powder was transferred to a 1.5 ml microtube. DNA was extracted from remaining rosary pea powder using plant DNA extraction protocol on Integrated DNA technologies (IDT) according to Ligler *et al*., [10].

Primer and PCR design

The reference sequences of the abrin toxin encoding gene in rosary pea was downloaded from NCBI [11]. We developed the primers for PCR amplification assays in order to detectthe abrin gene in samples suspected to bioterrorism. They were designed to produce a 328 bp PCR product. Primer3-plus software was used for primer design [12]. The OligoAnalyzer tool available at IDT website was used to analyze physical characteristics of primers' sequence, including GC content, melting temperature range, and self and hetero dimer probability [13]. Primers'sequence shown in Table 1, synthesized by Macrogen, South Korea.

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To evaluate the primers' performance and specificity, in NCBI website, Nucleotide BLAST service was used. Polymerase chain reaction was performed using Taq DNA Polymerase, 2x Master Mix RED 1.5 mM $MgCl_2$ (AMPLIQON, Denmark Cat.No. A180301). For maximum yield and specificity, temperatures, cycling time and primer concentration was optimized as shown in Table 2. A three-step PCR was run as a single PCR containing 0.5 pM final concentration of ach primer (see also primers in table 1), performed on the Eppendorf Mastercycler EP Gradient S Thermal Cycler (Eppendorf, Germany). According to manufacturer instruction, agarose gel electrophoresis and Simplysafe (EURx, Poland) staining were used to confirm PCR products.

Table 1. Primers' oligonucleotide sequence used in this study.

Primers	Sequences	bp
abrin forward	5′-AGCAATGGATGGGCTAGTCA-3′	20
abrin reverse	5′-TGGCGTAGGGTCTTGCTAAA-3′	20

Table 2. Three step PCR cycles' temperature and time.

	Step	Temp	Time	Number of cycles
Step1	Initial denaturation	95°C	5min	1
Step2	Denaturation	94°C	30s	40
	Annealing	56°C	30s	
	Extension	72°C	40s	
Step3	Final extension	72°C	5min	1

Sensitivity and capability in direct detection

To acquire acceptable sensitivity in the assay, a positive sample validated by agarose gel electrophoresis and sequencing, was purified using QIAquick PCR Purification Kit (QIAGEN, Germany). Purified PCR products were quantified using photometrical techniques and diluted in serial of $\log_{10}$ and amplified with the PCR method. In order to determine the gene copy number, we used the formula (Equation 1) in URI 'Genomics & Sequencing Center' website, created by Andrew Staroscik (2004) [14]. Also, a direct PCR was performed on a crude extraction of grinded seeds. For assessment of PCR inhibitors in a food matrix, DNA extracted from 0.3 g wheat flour, containing 1 mL crude extract was tested by PCR looking for the gene of interest.

Eq. 1

$$\text{Number of copies} = \frac{\text{amount}_{ng/\mu l} \times 6.022 \times 10^{23}}{\text{length}_{bp} \times 1 \times 10^{9} \times 650\ (\text{g/mol of bp})}$$

Results

Using optimized conditions as described above (Table 2), results of the current study revealed that designed primers sequence is a specific primer without interaction. The desired PCR product of 328 bp obtained from abrin gene and confirmed by agarose gel electrophoresis (Fig. 1). For validation of results, sequencing was performed for positive PCR products (Macrogen, South Korea) showing >90% similarity with abrin reference gene (X55667 abrin) (Fig. 2). Sensitivity analysis showed 1.22×101 copy numbers of PCR product per reaction (Table 3, Fig. 3). DNA samples extracted from crude extract, wheat flour containing abrin crude extract, and grounded seeds sample were also amplified by this assay and despite the presence of polyphenols and polysaccharides in wheat flour, no PCR inhibition was observed in this experiment.

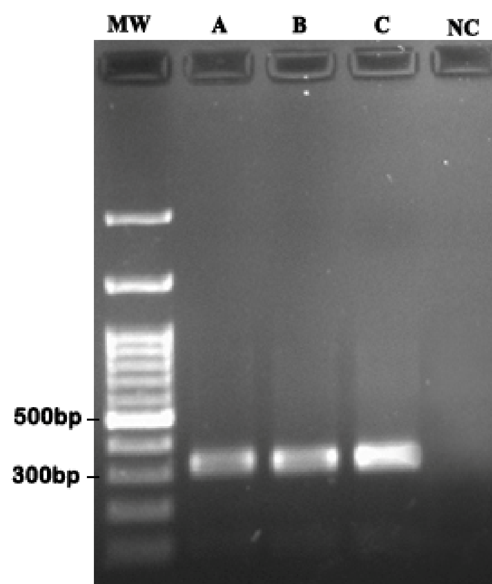

Figure 1. 328 bp of Abrin gene target in 1.5% agarose gel electrophoresis; MW: 100 bp DNA ladder; A: Direct PCR from grounded rosary pea seed; B: Direct PCR from Abrin crude extract; C: Wheat flour containing Abrin crude extract (DNA extracted by CTAB-PVP method describe above); NC: negative control.

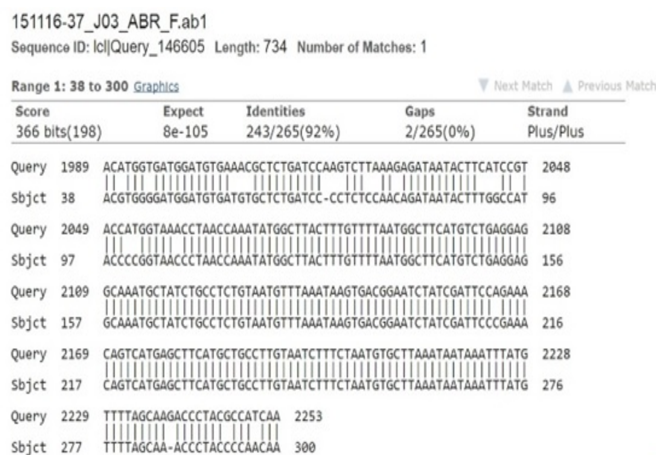

Figure 2. Alignments of PCR product sequencing result and X55667.1 *A. precatorius* gene for preproabrin.

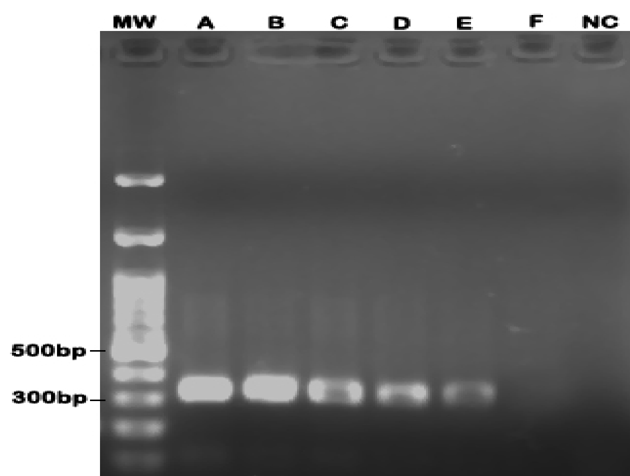

Figure 3. 1.5% agarose gel electrophoresis of $\log_{10}$ dilutions. MW: 100 bp DNA ladder; NC: negative controls; A, B, C, D, E, F: respectively, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11} dilution from 43.1 ng/µl purified abrin PCR product. Minimum detectable copy number for abrin gene in 10^{-10} dilution was about 12 copy.

Discussion

As the potential threats of bioterrorism by using these toxins seems to be increasing [5, 15], detection of these toxins have been extensively studied via different methods. There are several reported assays in recent years on the detection of abrin with the aim of efficiently and quickly abrin detection. Among these, as the immunological techniques have developed, antibody-based immunological methods were considered as an efficient method to be used for detection different abrin suspected samples. Garber *et al*., in 2008 reported an enzyme-linked immunosorbent assay and electro-chemiluminescence technology for detection of abrin in food [16]. Colloidal gold-based immune-chromatographic test strip for rapid detection of abrin in food samples were reported by Gao *et al*., (2012) that was able to detect 3 ng/ml abrin when it was directly spiked into milk. However, the test strip had low detection capability compared tothe usual methods of ELISA [17]. In 2014 Cho *et al*., reported a chromogenic enzyme-based sensor for detection of abrin poison in mock urine which was capable of detecting L-abrin as marker for abrin toxinin urine [18]. Xu *et al*., reported an ELISA and Colloidal Gold-Polyclonal Antibody Conjugate Immunochromatographic Assay for abrin detection which had a detection limit of 10 ng/mL [19]. However, it is clear that ELISA methods are time consuming and exposed to potential difficulties such as denaturizing due to sensitivity to temperature and low shelf life. Also, antibody generation for toxins has many disadvantages; for example, protein toxins may get lost or denatured. Therefore, a PCR assay with specific primers for abrin toxin gene was designed which showed competence of reliably detection of abrin gene indicating the presence of poison in samples. Since obtaining purified toxins requires advanced laboratory equipment and complex procedures, it is hypothesized that the perpetrators of such crimes are obliged to use the crude extracts [20]. Therefore, the remaining of specific-toxins gene in these extracts help investigators to benefit PCR as a detection technique to identify toxin gene and illustrate the presence of relative toxin in a suspected sample. This method can be used as the first line detection method or as avalidation for unsure ELISA positive or negative results. Additionally in the present study, using the CTAB-PVP DNA extraction method effectively provided polysaccharides and polyphenols-free DNA samples and abrin gene was detected in wheat flour containing abrin crude extract that contains inhibitory substance like food matrix.

Conclusion

According to the results of this study, a reliable method have been reported here, composed of single PCR assays, for detection this poisonous and important plant toxin. This method provides a sensitive, specific, and low-cost protocol for detecting a very important biological toxin gene in cases of suspicion to a bioterrorism event. The primers tested in this study were well designed in all parameters, so they can be used for multiplex PCR assays and Real-time PCR for detection of abrin toxin gene.

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