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Short Report

Rapid DNA Extraction Protocol from Stool, Suitable for Molecular Genetic Diagnosis of Colon Cancer

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ABSTRACT

Background: Colorectal cancer (CRC) is one of the most common forms of cancers in the world and is curable if diagnosed at the early stage. Analysis of DNA extracted from stool specimens is a recent advantage to cancer diagnostics. Many protocols have been recommended for DNA extraction from stool, and almost all of them are difficult and time consuming, dealing with high amount of toxic materials like phenol. Their results vary due to sample collection method and further purification treatment. In this study, an easy and rapid method was optimized for isolating the human DNA with reduced PCR inhibitors present in stool. **Methods:** Fecal samples were collected from 10 colonoscopy-negative adult volunteers and 10 patients with CRC. Stool (1 g) was extracted using phenol/chloroform based protocol. The amplification of P53 exon 9 was examined to evaluate the extraction efficiency for human genomic targets and also compared its efficiency with Machiels *et al.* and Ito *et al.* protocols. **Results:** The amplification of exon 9 of P53 from isolated fecal DNA was possible in most cases in 35 rounds of PCR using no additional purification procedure for elimination of the remaining inhibitors. **Conclusion:** A useful, rapid and easy protocol for routine extraction of DNA from stool was introduced and compared with two previous protocols. *Iran. Biomed. J. 11 (3): 203-208, 2007*

Keywords: DNA Extraction, Stool, Colon Cancer

INTRODUCTION

Colorectal cancer (CRC) is one of the most common forms of cancer in the world and is curable if diagnosed at an early stage [1]. Interest in screening has increased in recent years but it still remains low. Therefore, establishment of new screening programs is a priority.

Extensive research over the past 15 years has shown that a specific series of genetic changes (K-ras, P53, APC, MMR mutations) drives the neoplastic transformation of normal colonic epithelium to benign adenomas and subsequently to malignant adenocarcinomas [2]. The discovery of these genetic alterations has raised the possibility of detecting CRC through examination of the stool DNA because a healthy adult excretes approximately 10^{10} epithelial cells every day. Therefore, molecular examination of the genetic composition of the colonic mucosal cells, which are exfoliated into the stool, brought new ideas for CRC screening. Several attempts to determine major genetic changes in stool made a sensitive and specific panel to detect cancerous changes [3-6].

However, numerous technical problems remain to be resolved before initiating clinical trials using this approach. These problems include the low yield of DNA extracted from stool, which vary due to collection and extraction methods and presence of many DNA polymerase inhibitors like polysaccharides (from mucus, bacteria and food debris) and bile salts. In order to establish a genetic diagnostic measurement for colorectal tumor screening, several groups have attempted to improve

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DNA quality by testing several purification and amplification methods and to develop more sensitive assays for identifying gene [7-10]. Many protocols have been recommended for DNA extraction from stool. However, these protocols are difficult and time consuming, utilizing high amount of toxic materials like phenol, and complex procedures like absorptive columns and sometimes detergents like CTAB (cetyl trimethyl ammonium bromide) [9, 10]. Commercial kits are available too; however, they are expensive and sometimes unavailable.

In this study, we have developed rapid and easy procedure to isolate sufficient high quality DNA suitable for PCR amplification. The new procedure uses a modified Phenol/Chloroform extraction method. P53 was used as a target gene to analyze the efficiency of this method in producing high-quality DNA for PCR amplification and compared it with two other conventional extraction methods that were reported for DNA stool-based testing and described by Machiels *et al.* [9] and Ito *et al.* [10]. These protocols were selected because they seemed easy, without application of commercial kits and any expensive device or chemicals.

This protocol produces a sufficient amount of DNA for PCR amplification of human targets especially DNA composition of the tumoral colonocytes that contributes to only a small fraction of the human stool DNA. The tumoral DNA can be utilized for amplification of a potential marker in CRC screening.

MATERIALS AND METHODS

Human stool samples were collected from 20 individuals including 10 colonoscopy-negative adult volunteers without any dietary restrictions or antibiotic treatment and 10 patients with CRC. About 5 g stool was collected from each individual. All the samples were collected in dry clean plastic containers. Informed consent was obtained from every subject prior to the study. Stools were collected prior to any preparation for colonoscopy or 4-5 days following this procedure. The stool specimens were stored at -20°C immediately after collection, to avoid potential enzymatic degradation of nucleic acids, and then transferred to -70°C until use.

For DNA extraction, 1 g stool, frozen at -70°C, diluted in 10 mL of lysis buffer (Tris-HCL, 0.5 M; EDTA, 20 mM; NaCl, 10 mM; SDS, %0.1; pH 9.0) (TEN-9) in 50 mL tube. After vortexing for 5 minutes, samples were homogenized by shaking for 10 minutes. Samples were then diluted again (1/2) with 10 mL lysis buffer and homogenized for 5 minutes. Particulate materials were removed by centrifugation at 4500 ×g for 10 min. After transferring the supernatant to a new tube, approximately 10 mL of supernatant, DNA was precipitated by adding 5 ml ammonium acetate 7.5 M (half of the sample volume) and 25 ml of ice-cold ethanol 95-100% (twice the sample volume). Incubation at -20°C for 20-30 minutes will render a better precipitation. DNA was collected following centrifugation at 4500 ×g for 15 minutes at room temperature. In this step, precipitated DNA is not colorless and contains the bile salts. The DNA pellet was re-suspended in 600 µl of TE (pH 8) and incubated at 65°C for 15 minutes. Then, DNA was extracted organically and also purified using conventional single step phenol/chloroform/ isoamylalcohol protocol. Phenol would solve the colorful materials. After isopropanol precipitation, the colorless DNA pellet was collected and dissolved in 300 µl of Tris-EDTA buffer following an overnight incubation at 37°C.

These samples were also extracted using two other conventional methods as described by Machiels *et al.* [9], using CTAB and phenol/chloroform to remove inhibitors and Ito *et al.* [10], using Proteinase K, CTAB and phenol/chloroform to remove contaminants to perform a comparative analysis in efficiency of these methods.

The DNA yielded with each protocol was measured using spectrophotometer (BranTech Science, England); the amount of DNA was calculated in µg/ml by absorbance at 260 nm and the purity was tested by determining the 260/280 nm ratio. The efficiency of extraction protocols was analyzed using the same amount of each DNA (200 ng) by amplification of exon 9 of P53 as an amplification target. Primers were previously described by Beroud and Soussi [11].

[Forward: 5GCAGTTATGCCTCAGATTCAC3 and reverse: 5 AAGACTTAGTACCTGAAGGGT 3 ′]. PCR reaction mixture consisted of 1 X CinnaGen PCR buffer: each PCR primer, 500 nM; $MgCl_2$, 1.5 mM; dNTPs, 200 µmol/L and Taq DNA Polymerase, 1 U (CinnaGen, Tehran, Iran). Extracted DNA (200 ng) with each protocol was used in a reaction volume of 20 µL. PCR conditions were as follows: 3 min at 95 °C followed by 35 cycles of 50 s at 95°C, 120 s at 58°C and 120 s at 72°C followed by 72°C for 5 min as final extension, with maximum heating and cooling settings in

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Techne Thermal Cycler (Techgene, UK).

Amplified products (5 microliters) were electrophoresed through 2% agarose gel, and stained with ethidium bromide. P53 exon 9 amplification was analyzed through detection of the 138 bp band. DNA extracted from blood was used as the positive control. In this step, the number of amplifiable samples with each protocol was considered. Band intensity was measured using Kodak Image Analyzer software 1.0. It also used to compare the amount of PCR yield in normal and patient cases and also between different protocols. We also compared the time duration and amount of toxic materials used in each protocol.

RESULTS

The results of extraction with different methods in normal and tumoral samples are shown in Table 1. Various characteristics of the extraction methods including toxic materials, average DNA yield, DNA purity and range of band intensity are shown in Table1. The needed amount of stool for extraction procedure was 1 g in our protocol, 2 g in Machiels *et al.* [9] method and 100 mg in Ito *et al.* [10] protocol. Our extraction procedure is a single-day protocol and would not take more than 4 hours, in comparison with Machiels *et al.*[9] method that takes 7 hours and 3-day extraction protocol of Ito *et al.* [10]. As shown in Table 1, our protocol consumes less toxic materials in comparison with others; only single step phenol/chloroform purification would eliminate impurities. About 1.2 ml phenol/chloroform is needed for removing PCR inhibitors; however, Machiels *et al.* [9] method needs 4 times more phenol/chloroform and Ito *et al.* [10] consumes 5 times more toxic materials to produce amplifiable DNA. The average yield of DNA using our protocol measured about 143 µg in normal samples and 151 µg in patients with CRC using 1 g stool. Other protocols yielded a lower amount of DNA in the same portion of stool sample (Table 1). The average yield of DNA in tumoral samples was more than normal. Measuring the DNA purity by calculating 260/280 ratio showed that all protocols produce suitable DNA (ratio 1.8-2) for PCR amplification in theory.

Table 1. Comparison of efficiency of different protocols.

Protocol		Our Protocol	Ito *et al.*	Machiels *et al.*
Amount of stool needed		1 g	100 mg Day 1:10 min	2 g
Duration		4 h	Day 2: 1 h Day 3: 4 h	7 h
CTAB Purification		-	+	+
Phenol/chloroform purification steps		1	2	2
Additional chloroform purification steps		0	2	2
Amount of toxic Materials	Phenol	0.6 ml	2 ml	4 ml
	Chloroform	0.6 ml	2 ml	2 ml
Average DNA yield (µg/ g stool)	C	143	64.8	68.8
	P	151.8	67	100
Range of DNA purity (260/280)	C	2	1.96	2.6
	P	1.97	1.8	1.9
Number of amplifiable samples	C	8/10	5/10	6/10
	P	10/10	7/10	8/10
Range of band intensity (ng)*	C	7	6	10
	P	106	85	130

C, Healthy control; P, patient with cancer; *measured by Kodak Image Analyzer software 1.0.

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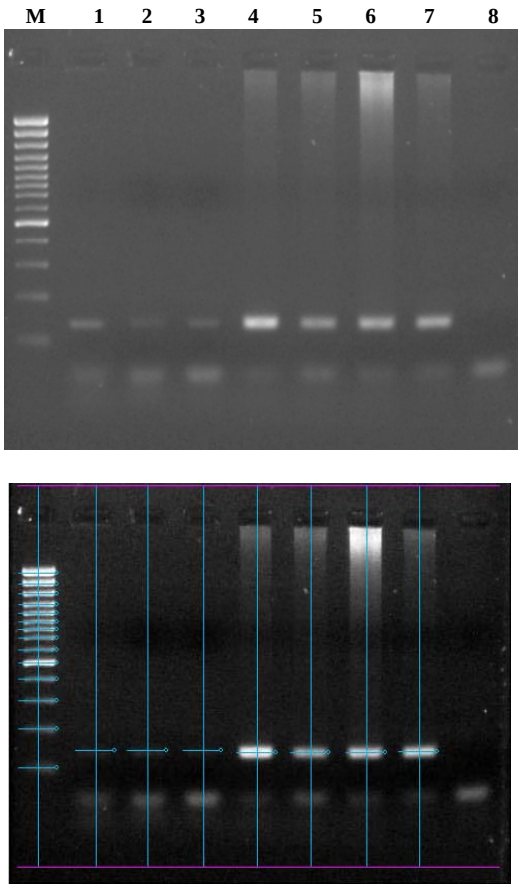

Fig. 1. Comparison in amplification of Exon 9 of TP53 using Stool DNA extracted from one of normal individuals and one of CRC affected Patients. M, 100 bp DNA ladder (Fermentas, Lithuania); Lane 1, normal, Machiels *et al.* protocol; Lane 2, normal, Ito *et al.* protocol; Lane 3, normal, our protocol; Lane 4, patient, Machiels *et al.* protocol; Lane 5, patient, Ito *et al.* protocol; Lane 6, patient, our protocol; Lane 7, blood sample as positive control; Lane 8, negative control; And at the right hand, band integrity analysis of the samples was shown that is prepared by Kodak Image Analyzer software 1.0.

Amplification of P53 exon 9 was performed to evaluate extraction efficiency for human targets. The number of amplifiable samples was measured in each protocol. Although DNA ratios seemed acceptable for PCR amplification, P53 was amplified in 8 out of 10 normal individuals and 10 out of 10 CRC cases using our protocol. In Machiels *et al.* [9] method 6/10 of normal and 8/10 patient samples and 5/10 of normal and 7/10 patients in Ito *et al.* [10] were amplifiable. In samples, PCR would not render a band, applying 1/2-1/5 dilution of DNA samples lead to amplification. The band intensity for PCR products was shown to be stronger in Machiels *et al.* [9] method Using Kodak Image Analyzer software 1.0 (Table 1). Differences in the efficiencies of PCR reactions for stool DNA from CRC patients and disease-free individuals were observed in all protocols. Band intensities for PCR products amplified from stool DNA of CRC patients were higher than the intensities for PCR products of normal cases (Fig. 1).

Ultimately, the results showed that our method produces adequate high quality of human DNA from stool samples suitable for amplification of P53 gene in all patients using small amount of stool sample as low as 1 g.

DISCUSSION

Although calculated 260/280 ratio was acceptable in most samples, not all samples showed a PCR product suggesting the presence of impurities that inhibit PCR amplification. Therefore, procedures of DNA purification from stool samples must be carried out to exclude these impurities. Feces constitute complex biological samples which cause problems when PCR is used as a diagnostic method, not only because of the presence of numerous types of bacteria but also because of the different kinds of food degradation products present in the stool [12]. Most of these food products (e.g. polysaccharides, lipids, hemoglobin) exhibit similar solubility to DNA. As a consequence, they are not completely removed during classical extraction protocols (such as detergent, protease and phenol/chloroform treatments), remaining as contaminants in the final DNA preparations which reduces the DNA purity and in turn work as strong PCR inhibitors.

Several methods have been developed to avoid these DNA impurities from stool. In methods described by Vogelstein and Kinzler [7], a stool lysis buffer is used to lyse eukaryotic cells for the subsequent separation of the bacteria. Deuter *et al.* [8] tried to separate the inhibitors by using an absorption matrix with carbohydrates and colestipolhydrochloride. Machiels *et al.* [9] obtained reproducible template DNA from the stool, by using CTAB purification in addition to the conventional DNA extraction. CTAB purification was taught to eliminate the presence of polymerase inhibitors [13]. Although CTAB reduces the impurities of DNA, like phenol, it also reduces the final yield of DNA.

In our procedure, lack of PCR products in some samples suggests that PCR inhibitors were present in extracted DNA. To prove this, we mixed human DNA extracted from blood with DNA extracted from stool in different ratios and analyzed the

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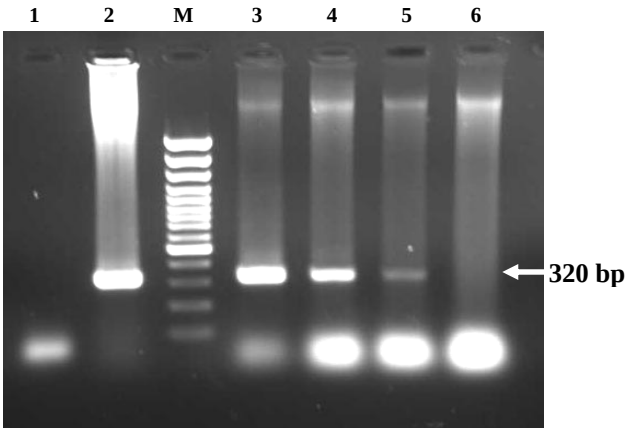

Fig. 2. The inhibitors present in stool inhibited amplification of Blood DNA. Dilution effect in removing inhibitors from amplification reaction of β-actin PCR. Lane 1, negative control; Lane 2, positive control (0.5 µl blood DNA); Lane M, 100 bp DNA ladder (Fermentas, Lithuania); Lane 3, (0.5 µ1 stool DNA + 0.5 µl blood DNA), maximum band intensity, Lane 4, (1 µl stool DNA + 0.5µl blood), the bands intensity decreased; Lane 5, (2 µl Stool DNA + 0.5 µl blood DNA), weak amplification and Lane 6, (3 µl stool DNA + 0.5 µl blood DNA) no amplification.

amplification of β-actin with this mixed DNA. The result illustrated that with adequate dilution of DNA extracted from stool the intended segment would amplify more easily (Fig. 2).

For testing the dilution effect, we diluted the sample from 1:2 to 1:10 ratios. For some samples, a simple 1:2 dilution was sufficient to eliminate the inhibitors, while for others, a 1:10-dilution was necessary. This suggests that inhibitors are not present in all feces, and their concentration is not constant in all samples (The data not shown). In general, the result showed adequate dilution will thin the impurities from extracted DNA and make it suitable for PCR amplification.

Measuring band intensities revealed a significant difference in normal and tumoral samples. This result suggests that the amount of human DNA in feces may be increased in individuals with CRC. Villa *et al.*, [14] found that ß-globin sequences were amplified by PCR more frequently in patients with either colorectal carcinoma or adenomas than in healthy individuals. Kelaassen *et al.*, [15] demonstrated increased amounts of human DNA in the feces of patients with colorectal tumors compared with healthy persons. The difference in PCR efficiency is related to the presence of a greater concentration of human DNA in the stools of CRC patients. Boynton *et al.*, [16] described that the increased concentrations of human DNA could be explained by decreased apoptosis of bowel cells and/or increased shedding of cancer cells or inflammatory cells into the colonic lumen.

This protocol can be used as an effective and reproducible alternative to other long extraction protocols, with the additional advantage of avoiding considerable DNA losses and the use of extra volume of toxic and expensive materials. It has fewer steps and manipulations, thus reduces the risk of contamination with foreign DNA. Furthermore, no further purification treatment needed and crude stool is used and no special pretreatment of patient samples is necessary. The amplification of single-copy human genes from isolated fecal DNA was possible in almost all cases after 35 rounds of PCR. In cases where inhibitors persisted, diluting (1:2-1:5) was sufficient to remove their effects. In spite of the presence of inhibitors, this protocol was developed using no purification procedure for eliminating the remaining inhibitors.

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