

Evaluation of the Efficacy of Five DNA Extraction Methods for the Detection of *Mycobacterium tuberculosis* DNA in Direct and Processed Sputum by an In-House PCR Method*

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Aim: DNA extraction is an important step from clinical samples for molecular diagnosis of tuberculosis by PCR. The aim of this study was to evaluate the efficacy of five DNA extraction methods [boiling, single step proteinase K, GuSCN lysis and izopropanol precipitation (Heliosis, METİS Company, TURKEY), DNA precipitation (Epicentre Technologies), and solid phase absorption (QIAamp DNA mini kit, QIAGEN, Valencia,CA)] in searching *Mycobacterium tuberculosis* DNA in smear positive sputum samples.

Materials and Methods: A total of 50 sputum samples were extracted directly and after digested with 4% NaOH-NALC methods using 5 DNA extraction methods. All DNA extracts were studied by an in-house PCR method.

Results: The rate of the positive detection for 5 extraction methods was 22% with boiling method, 38% with single step proteinase K, 38% with guanidium isothiocyanate lysis and isopropanol precipitation method (Heliosis, METİS), 42% with DNA precipitation (Epicentre Technologies), and 58% with solid phase absorption (QIAamp).

Conclusions: When the rate of positive detection is taken into consideration in smear positive patients, solid phase absorption method seems to be more proper to use routinely for DNA isolation from clinical samples.

Key Words: DNA extraction, *Mycobacterium tuberculosis*, PCR

Direk ve İşlenmiş Balgam Örneklerinde In-House PCR Yöntemi ile *Mycobacterium tuberculosis* DNA'sının Tanısı İçin Beş DNA Ekstraksiyon Yönteminin Etkinliğinin Değerlendirilmesi

Amaç: DNA ekstraksiyonu klinik örneklerde PCR yöntemi ile tüberkülozun moleküler tanısında önemli bir basamaktır. Bu çalışmada, yayma pozitif balgam örneklerinde *Mycobacterium tuberculosis* DNA'sının araştırılmasında kaynatma, tek basamaklı proteinase K, GuSCN lysis ve izopropanol presipitasyon (Heliosis, METİS Company, TURKEY), DNA presipitasyon (Epicentre Technologies) ve katı faz absorpsiyonu (QIAamp DNA mini kit, QIAGEN, Valencia,CA) olmak üzere beş farklı ekstraksiyon yönteminin etkinliğinin değerlendirilmesi amaçlanmıştır.

Materyal ve Metod: Toplam 50 adet balgam örneği direkt olarak ve % 4 NaOH- NALC metodu ile muamele edildikten sonra beş farklı DNA ekstraksiyon yöntemi ile ekstrakte edildi. Tüm örnekler In-House PCR yöntemi ile çalışıldı.

Bulgular: Ekstraksiyon yöntemlerine göre pozitiflik saptama oranı; kaynatma metodu ile % 22, tek basamaklı proteinase K ile % 38, GuSCN lysis ve izopropanol presipitasyon (Heliosis, METİS) ile % 38 ve DNA presipitasyon (Epicentre Technologies) ile % 42 ve katı faz absorpsiyonu (QIAamp) ile % 58 olarak bulundu.

Sonuç: Yayma pozitif hastalarda pozitif saptama oranı dikkate alındığında katı faz absorpsiyon metodunun rutin kullanımda klinik örneklerden DNA izolasyonu için daha uygun bir yöntem olduğu kanısına varılmıştır.

Anahtar Sözcükler: DNA ekstraksiyonu, *Mycobacterium tuberculosis*, PCR

Introduction

Polymerase chain reaction (PCR) has been widely used for rapid diagnosis of *Mycobacterium tuberculosis* complex from clinical samples. Recently, there have been many kinds of in-house PCR methods and commercial detection kits used with the aim

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of routine diagnosis (1-4). The sensitivity of these methods changes according to the DNA extraction method, target region, and probe using. To date, extensive clinical studies have shown that there is no method that can be alternative to the conventional methods to get the same sensitivity and specificity yet. It has been believed that the sensitivity of these tests that require a good laboratory equipment and experience could be affected by fundamental PCR steps, such as the type of the clinical sample, the presence of the inhibitory factors in the sample, DNA isolation (amplification and imaging procedures) in addition to homogenization-decontamination processing (5-9). This study has been carried about using 5 DNA extraction techniques in sputum samples collected from the smear positive patients with prediagnosed with tuberculosis to investigate the performance of an in-house PCR method in detecting positive results.

Materials and Methods

In our study, sputum specimens collected from 50 untreated-hospitalized patients, who were detected pulmonary tuberculosis by clinical and radiological findings, and the sputum specimens including bacterial suspensions in 10^6 , 10^4 , 10^2 , and 10^1 cfu/ml density, prepared from *Mycobacterium tuberculosis* H₃₇Ra standard strain, were evaluated. Bacterial suspensions were prepared into sputum samples collected from healthy people, not suspected of having tuberculosis, whose negativities were confirmed by PPD test and AFB staining. By dividing 2 for each dilution, the specimens were evaluated before and after processed by 4% NaOH-NALC procedure (10).

The sputum specimens taken from the patients and the bacterial suspensions were decontaminated and homogenized by 4% NaOH-NALC procedure. For DNA isolation, each 1 ml from prepared main suspension was poured into 5 different microcentrifuge tubes. These samples were stored at -20 °C for later analysis with molecular methods. The rest of each samples were inoculated onto Lowenstein-Jensen (LJ) culture medium and used for Erlich-Ziehl Neelsen (EZN) staining. Eight weeks later, the culture tubes, which were incubated at 37°C, were checked, and the tubes with no growing were labeled negative. The identification of *M. tuberculosis* in specimens with growth was made by nitrate reduction,

niacin, tiofen 2 -carboxylicacide hydrazide (TCH), and paranitrobenzoic acid (PNB) tests (11-15). DNA isolation was made by 5 different extraction methods: boiling after processing homogenization-decontamination and concentration by 4% NALCNaOH method, solid phase absorption (QIAamp DNA mini kit, QIAGEN, Valencia,CA), single step proteinase K, guanidium Isothiocyanate lysis and isopropanol precipitation (Heliosis, METİS,Turkey), and DNA precipitation (Epicentre Technologies, MADISON WI) (5,6). DNA specimens were amplified by an in-house PCR method by using primers, INS 1: 5' – CGT GAG GGC ATC GAG GTG GC- 3' and INS 2: 5' – GCG TAG GCG TCG GTG ACA AA- 3', which were amplified the region 245 base pair on IS6110 region existing in the genome of *Mycobacterium tuberculosis* complex. PCR products were run by 2% agarose gel electrophoresis processing. After staining with ethidium bromide, the gel was evaluated using DNA molecular weight standard (f X174 Hae III) on a UV transilluminator. The products that were detected 439 base pair-DNA band were accepted as positive. In the study, *Mycobacterium tuberculosis* H₃₇Ra strain was selected as a positive control, and the negative control was distilled water.

Results

In this study, 10^6 , 10^4 , 10^2 , and 10^1 dilutions were evaluated by 5 different extraction methods, before and after homogenization and decontamination were processed by standard 4% NaOH-NALC method (Table 1).

In the evaluation made by direct and processed methods, PCR was positive in all extraction methods in 10^6 dilution. In 10^4 dilution, PCR was found positive in the other 4 extraction methods, except for boiling. In 10^2 dilution, while PCR was positive in extraction performed from direct samples by solid phase absorption method (QIAamp), in the same dilution, the result was negative in the processed samples. In 10^1 dilution, the results of all the PCR studies in direct and processed samples by 5 extraction methods were negative.

Fifty sputum samples tested in the study, after processed by 4% NaOH-NALC method, were assessed with EZN staining. Clinical samples were divided into 4 groups by the scale of the microscopic evaluation (Table 2).

PCR results were compared to clinical findings and the culture (Lowenstein-Jensen) method. The culture method

Table 1. In-house PCR results belonging to dilutions of *Mycobacterium tuberculosis* H37 Ra strain by 5 different DNA extraction methods.

DNA Extraction Methods	Dilutions							
	10 ⁻⁶		10 ⁻⁴		10 ⁻²		10 ⁻¹	
	Direct	4% NaOH-NALC	Direct	4% NaOH-NALC	Direct	4% NaOH-NALC	Direct	4% NaOH-NALC
Boiling	+	+	-	-	-	-	-	-
Single step proteinase K	+	+	+	-	-	-	-	-
GuSCN lysis ve izopropanol precipitation (Heliosis, METIS)	+	+	+	-	-	-	-	-
DNA presipitation (Epicentre Technologies)	+	+	+	-	-	-	-	-
Solid phase absorption (QIAamp)	+	+	+	+	+	-	-	-

Table 2. In-house PCR results by the scale of the microscopic evaluation (AFB staining) of patient sputum samples.

DNA Extraction Methods	The groups of the sample				
	Smear (-) (n=8)	Smear (+) (n=11)	Smear (++) (n=17)	Smear (+++) (n=14)	Total (n=50)
Boiling	-	-	4 (23.52%)	7(50%)	11 (22%)
Single step proteinase K	-	-	5(29.41%)	14 (100%)	19(38%)
GuSCN lysis ve izopropanol precipitation (Heliosis, METIS)	-	1(9.09%)	8(47.05%)	10(71.42%)	19(38%)
DNA precipitation (Epicentre Technologies)	-	-	7 (41.17%)	14(100%)	21(42%)
Solid phase absorption (QIAamp)	-	4 (36.36%)	14 (82.35%)	11(78.57%)	29(58%)

N = number of samples

was positive in the 46 of 50 samples which were prediagnosed from tuberculosis with clinical and radiological findings. The samples, whose cultures were negative, were in the patient group whose smears were negative. PCR results of smear-negative patients were negative by all 5 extraction methods. In the group of smear positive, 1 sample by GuSCN lysis and Isopropanol precipitation method (Heliosis, METIS), and 4 samples by solid phase absorption method were positive by PCR. The average rate of the positive detection of 5 extraction methods in all the samples was 22% with boiling method, 38 % with guanidium Isothiocyanate lysis and isopropanol precipitation method (Heliosis, METIS), 42% with DNA precipitation (Epicentre Technologies), and 58% with solid phase absorption (QIAamp). (Table 2).

Discussion

One third of the world population is infected with tuberculosis and approximately 8 million new cases are reported every year (16). The main goal of Tuberculosis Control Programming is to detect and treat the sputum smear positive-patients without delay. Clinical and radiologic findings play an important role in diagnosis of tuberculosis infections. The precise diagnosis of tuberculosis is maintained by microbiologic methods. It is accepted as a gold standard that the agent is demonstrated first by examining stained smears through a microscope, and then by culturing the organisms on the medium. However, the precise diagnosis is delayed because the sensitivity of the microscopy method is poor and the incubation period takes 2-8 weeks for the diagnosis by culture. Recently, molecular methods to

detect mycobacteria have been used widespread in laboratories to support conventional methods (9, 17, 18). The sensitivity of these methods depends on clinical specimen type, homogenization-decontamination processing, and the principles of the PCR method. The most important factor, whose sensitivity is crucial, among PCR test procedure steps is the DNA extraction from clinical specimens. It has to isolate an accurate DNA sample that does not include the inhibitory substances and is purified from cell structures for a successful PCR test (19, 20). In our study, the extraction methods widely used for bacterial suspensions and smear positive samples in routine diagnostic laboratory were evaluated. As shown in Table 2, the rate of the positive detection of the extraction methods was found 22% by boiling method, 38% by single step proteinase K, guanidium Isothiocyanate lysis and isopropanol precipitation method (Heliosis, METIS), 42% by DNA precipitation (Epicentre Technologies), and 58% by solid phase absorption (QIAamp). In previous studies, the sensitivity of PCR changes between 91 and 97% in AFB positive-specimens, and between 40% and 74% in AFB negative specimens. But, specificity is detected between 77-100% in both groups (21-23).

Nolte and his colleagues reported that PCR sensitivity changed between 67-99% according to the score of AFB positive in their study performed by sputum specimens (24). Tevere et al. have determined that the sensitivity of PCR in sputum samples diagnosed clinically tuberculosis was 100% for AFB positive samples, and 73% for AFB negative samples (25). In our study, PCR examining in smear-negative 8 patients, who were prediagnosed

pulmonary tuberculosis by clinical and radiological findings, was found negative by 5 types of extraction methods. The results of the culture in 4 patients from this group were negative. In our study, when clinical diagnosis of the patients are taken consideration, it is concluded that the in-house PCR test that we applied by 5 types of extraction methods is not proper for diagnosis in smear negative patients. CDC suggests that PCR tests can be used in smear positive patients. In the study that Beige and his coworkers performed, PCR was positive in 100% of AFB positive and 94% of culture positive from the samples, and in 17% of those did not have tuberculosis (26). In another study on PCR test which was performed with Sputum samples, 67 (84%) out of 80 samples, which *M. tuberculosis* growing was observed, were positive by PCR test, and it is demonstrated that there is a strong correlation between the rate of AFB positivity and the sensitivity of PCR (27,28). In our study, in the group of smear positive (+) samples, 36.36% was detected positive by solid phase absorption (QiaAmp) method. This rate was 82.3% in the group of smear (++) samples, and 78.57% in the group of smear (+++) samples. In the group of smear (+++) samples, PCR was detected as 100% positive by using single step proteinase K, and DNA precipitation methods. It is observed that our results are concordant with other investigators' findings. Furthermore, it is striking that isolations of DNA in sputum samples using chemical methods are more effective in the PCR test. Among these methods, if the rate of positive detection is taken into consideration, in smear positive patients, solid phase absorption method is more proper to use routinely for isolation from clinical samples.

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