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Molecular detection of Mycobacterium tuberculosis in formalin-fixed, paraffin-embedded tissues and biopsies of gastrointestinal specimens using real-time polymerase chain reaction system

Formalinde fikse edilmiş, parafine gömülmüş gastrointestinal dokularda ve biyopsilerde gerçek zamanlı polimeraz zincir reaksiyonu sistemi kullanarak M. tuberculosis'in moleküler olarak tespit edilmesi

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Background/aims: *We aimed to perform the molecular detection of Mycobacterium tuberculosis in formalin-fixed, paraffin-embedded tissues and biopsies of gastrointestinal specimens using real-time polymerase chain reaction system.* ***Methods:*** *The study included three groups: (A) control (n=24), with no previous signs of M. tuberculosis complex (MTBC), (B) patients (n=28) with known TB origin and (C) patients (n=50) with clinical and histopathological signs of TB but who were culture- and acid-fast bacilli (AFB)-negative. The samples were obtained from the Medical and Surgical Gastroenterology Departments of Bhopal Memorial Hospital and Research Centre. We extracted DNA using DNeasy Blood & Tissue kit (QIAGEN, Germany) and performed real- time assay using Roche LightCycler 2.0 with fluorescence resonance energy transfer (FRET) hybridization probes obtained from Roche Molecular Diagnostics (USA) for the specific amplification of the 159 bp region of the mycobacterium genome.* ***Results:*** *All the samples (n=24) of Group A were found to be negative, while in Group B, 27 out of the 28 cases studied were found to be positive by LightCycler real-time polymerase chain reaction (LC PCR). In Group C, 18 out of the 50 cases studied were found to be positive, showing a positivity of 36%. The overall positive and negative predictive values of the test for clinical TB (Group C) were 100% and 96.9%, respectively.* ***Conclusions:*** *Results of our investigation demonstrated that the real-time detection technology using FRET probes has much higher sensitivity for the detection of MTBC DNA in tissue biopsy samples and formalin-fixed paraffin-embedded surgically resected tissues of the gastrointestinal tract.*

Key words: *Mycobacterium tuberculosis,* intestinal tuberculosis, real-time PCR, LightCycler, fluorescence resonance energy transfer (FRET) probes

Amaç: *Formalinde fikse edilmiş, parafine gömülmüş gastrointestinal dokularda ve biyopsilerde gerçek zamanlı polimeraz zincir reaksiyonlarını (GZ-PZR) kullanarak M. tuberculosis'in moleküler olarak tespit edilmesi amaçlanmıştır.* ***Yöntem:*** *Çalışmaya M. tuberculosis hikayesi olmayan 24 hasta (Grup A; kontrol), tüberküloz hikayesi olan 28 hasta (Grup B) ve kültür veya ARB negatif olan ancak tüberküloz kliniği olan 50 hastadan (Grup C) alınan gastrointestinal doku örnekleri dahil edilmiştir. Doku örnekleri Bhopal Memorial Hastanesi ve Araştırma Merkezinin medikal ve cerrahi gastroenteroloji kliniklerinde izlenen hastalardan elde edilmiştir. DNA ekstraksiyonu DNeasy Blood & Tissue kiti (QIAGEN, Almanya) ile, polimeraz zincir reaksiyonu işlemleri ise mycobakterinin 159 bazlık özgül bölgesine bağlanan floresan rezonans transfer hibridizasyon problarının (Roche Molecular Diagnostics, ABD) kullanıldığı Roche Light Cycler 2.0 sistemi ile gerçekleştirilmiştir.* ***Sonuçlar:*** *Grup A'dan alınan tüm örnekler (n=24) negatif bulundu. Buna karşılık grup B'de çalışılan 28 hastanın 27'sinde pozitiflik tespit edildi. Grup C'de 50 hastanın 18'inde (%36) polimeraz zincir reaksiyonu pozitiflik bulundu. Genel olarak polimeraz zincir reaksiyonunun pozitif prediktif değeri %100, negatif prediktif değeri %96,9 bulundu.* ***Tartışma:*** *Çalışmamızın sonuçları ortaya koymuştur ki, floresan enerji transfer probları kullanılarak gerçekleştirilen polimeraz zincir reaksiyonu işlemi, gastrointestinal sistemden alınmış formalin ile fikse edilmiş ve parafine gömülmüş doku örneklerinde M. Tuberculosis DNA'sının tespit edilmesinde oldukça duyarlı bir yöntemdir.*

Anahtar kelimeler: Mycobacterium tuberculosis, barsak tüberkülozu, gerçek zamanlı PCR, Light Cycler, FRET problar

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INTRODUCTION

Tuberculosis (TB), due to its high risk of morbidity and mortality, remains a major global public health problem. According to the World Health Organization, there are approximately 14.4 million prevalent cases and 9.2 million new cases being reported worldwide, with 1.5 million deaths in 2006, occurring mostly in developing countries. India contributed one-fifth of the global cases and 325,172 of the deaths in the 2005 cohort (1).

Depending on the immune status of the patient, this contagious disease not only infects the lungs (as pulmonary TB) but can also manifest in extrapulmonary sites of the body such as the gastrointestinal tract, peritoneum, lymph nodes, or solid viscera. The gastrointestinal tract remains a major extrapulmonary site for TB, as its incidence has been found to be increasing in the last decade (2).

The diagnosis of gastrointestinal TB remains a major challenge. It can be delayed since the signs and symptoms, such as tissue granuloma, might overlap with that of other infectious and non-infectious diseases, further resulting in increased morbidity and mortality (3, 4). Moreover, the traditional bacteriologic studies including the microscopic demonstration of acid-fast bacilli (AFB) and mycobacterial culture are time-consuming and lack sensitivity (5), thereby reinstating the need for faster and more effective diagnostic tools.

Diagnosis of *Mycobacterium tuberculosis*-specific DNA sequences represents a rapid and sensitive method (6). Conventional polymerase chain reaction (PCR) methodology like single- step PCR and nested PCR, though they provide a likely strategy, pose an enhanced risk of sample contamination instead (7). In this aspect, real-time PCR technology serves as an apt alternative, since PCR and fragment analysis are performed in a single closed tube, thereby minimizing the risk of contamination. In real-time PCR, the amplified product is detected via fluorescent dyes. These are usually linked to oligo-nucleotide probes that bind specifically to the amplified product. Monitoring the fluorescence intensities during the PCR run (i.e. in real time) allows the detection and quantification of the accumulating product without having to reopen the reaction tubes after the PCR run (8). In addition to conventional qualitative analysis, real-time PCR assays make it possible to perform accurate quantitative analyses with a high degree of reproducibility (9-11).

Real-time detection technology has made it possible to establish non-commercial, probe-based PCR systems that provide stable operation, low contamination risk and automated interpretation of results (12). Detection of *M. tuberculosis* complex (MTBC) in the extrapulmonary mode of the disease through these advanced diagnostic tools has not received the attention it is due. Thus, the using fluorescence resonance energy transfer (FRET) technology based on hybridization probe, we applied a novel technique consisting of an internally controlled quantitative real-time PCR assay that provided a significant improvement in detection sensitivity and quantification.

MATERIALS AND METHODS

Specimen Collection

The aim of this study was to test the efficacy of advanced molecular testing methodology such as real-time PCR (LightCycler [LC] 2.0) for the rapid detection of extrapulmonary MTBC from formalin-fixed, paraffin-embedded (FFPE) tissues and endoscopic biopsies. A total of 102 samples were included in the study, which were divided into three groups: (A) control (n=24), with no previous signs of MTBC infections, (B) patients (n=28) with known TB origin and (C) culture- and AFB-negative patients (n=50) suspected to be MTBC-positive as they showed clinical and histopathological signs of TB with no coexistence of pulmonary or human immunodeficiency virus (HIV) infections. The FFPE tissue and endoscopic biopsy samples were obtained from the Medical and Surgical Gastroenterology Departments of Bhopal Memorial Hospital and Research Centre and stored at -20°C (processed within 7 days).

The controls (Group A) included in the study were patients with no evidence of *M. tuberculosis* infection. The patients in Group B were known to be positive for intestinal TB based on gold standard microbiological AFB (Ziehl-Neelsen, ZN) stain and culture. The inclusion criteria of the patients in Group C were chosen according to the presence of certain symptoms, namely, abdominal pain, dyspepsia, weight loss, fever, anorexia, nausea and vomiting, constipation, rectal bleeding, chronic or bloody diarrhea, change in bowel habits, malabsorption, and additional suspicious lesions in other body parts. Upon histopathological analysis, specimens revealed typical chronic granulo-

matous inflammation with caseous necrosis, indicative of probable mycobacterial infection.

Microbiological Examination

The BacT/ALERT 3D system (bioMérieux Inc., NC, USA) was used for the culture-based detection of the MTBC. The method is based on the detection of carbon dioxide (CO_2) released by actively proliferating mycobacteria. The elevated CO_2 concentration lowers the pH in the medium, which in turn produces a color change in a sensor in the vial, which is detected by a reflectometric unit in the instrument. The BacT/ALERT automatically performs readings every 10 minutes (min), and all data are transferred to and saved in the BacT/VIEW data management system.

The classic ZN technique was used for the acid-fast staining; the smear is flooded with carbol-fuchsin dye, heated and then decolorized. The non-acid-fast organisms are then counterstained with methylene blue. Acid-fast MTBCs appear red due to the retention of the carbol-fuchsin while the background and any non-acid-fast organisms will appear blue due to the methylene blue counter stain.

DNA Isolation and Sample Preparation

For sample preparation, the FFPE specimens were sliced with disposable sterile blades in each paraffin block, and deparaffinized twice with 1 ml xylene and twice with 1 ml ethanol (100%) (13), whereas for the formalin-fixed tissue biopsy and endoscopy samples, the specimens were washed twice in phosphate buffered saline (PBS) prior to DNA isolation, and DNA was extracted from the samples by proteinase K digestion in combination with DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany), following the tissue protocol.

Real-Time MTBC Detection Assay

Real-time assay was performed in the thus extracted DNA with the help of FRET probes from Roche Molecular Diagnostics (USA) using Roche LightCycler 2.0. The working master mix was prepared as per the kit protocol by adding 13.5 µl master mix concentrate, 2 µl Mg^{2+} and 0.5 µl internal control. Finally, 15 µl of working master mix and 5 µl of the sample were dispensed in each capillary and were run on Roche LightCycler 2.0. Standard amplification parameters were as follows: one cycle of 95°C for 20 seconds (sec), followed by 45 cycles of 95°C for 01 sec, 63°C for 15 sec and 72°C for 15 sec and a single cooling cycle, i.e. 40°C for 20 sec. Real-time data were analyzed with LC software, version 4.0 (Roche Molecular Diagnostics, USA). The real-time assay uses primer binding sites that are conserved in all members of the MTBC. The generation of the specific amplicon (159 bp) is detected by a labeled fluorogenic probe.

RESULTS

A positive fluorescence signal in 5 out of 10 FFPE and 13 out of 40 tissue biopsies (Table 2) confirmed the presence of MTBC DNA in the patients presumed to be positive (Group C). An overall 36% positivity was found in these samples. However, the utility of real-time PCR assay in the known culture and ZN stain-positive specimens (Group B) showed 96.4% sensitivity in MTBC diagnosis, as 27 of all 28 samples reported in the study of this group were positive through LC PCR (Tables 1, 2). The quantitative bacterial load ranged from 1.62 x 10^{-6} to 5.46 x 10^{2} copies/µl (Table 3). The results observed in Group A (non MTBC) showed the specificity of LC PCR to be 100%, as no positive signal was observed in the total 24 studied cases. The internal control of the real-time PCR assay was positive for all specimens, negating the existence of possible inhibitions. With a positive PCR test, there is a 100% probability of having extrapulmonary TB (positive predictive value) with a minimum bacterial load of 1.6 copies/µl, and a negative PCR test gives a 96.9% negative predictive value, i.e., chance of truly ruling out a MTBC infection in the gastrointestinal tract (Table 1).

Table 1. Total bacterial load in LC PCR positive samples*

S. NO	Sample Type	Site of involvement	Bacterial DNA load (copies/µl)
1	FFPE	Ileum	1.46 x 10^{0}
2	FFPE	Abdomen	4.83 x 10^{0}
3	FFPE	Ileum	1.65 x 10^{1}
4	FFPE	Rectum	1.62 x 10-^{6}
5	Tissue Biopsy	Rectum	1.60 x 10^{0}
6	Tissue Biopsy	Ileum	5.44 x 10^{-1}
7	Tissue Biopsy	Ileum	7.05 x 10^{-1}
8	Tissue Biopsy	Cecum	6.07 x 10^{0}
9	Tissue Biopsy	Ileum	5.46 x 10^{2}
10	Tissue Biopsy	Ileum	5.82 x 10^{0}
11	Tissue Biopsy	Cecum	4.23 x 10^{0}
12	Tissue Biopsy	Cecum	2.51 x 10^{-1}
13	Tissue Biopsy	Cecum	6.20 x 10^{-1}
14	Tissue Biopsy	Colon	3.32 x 10^{0}
15	Tissue Biopsy	Rectum	2.48 x 10^{0}
16	FFPE	Liver	3.98 x 10^{0}
17	Tissue Biopsy	Cecum	1.25 x 10^{-2}
18	Tissue Biopsy	Rectum	3.92 x 10^{-1}

*FFPE: Formalin-fixed paraffin-embedded tissue. LC PCR: Light Cycler polymerase chain reaction.

Table 2. Results of real-time PCR under various categories

Category n (%)	Total	Real-Time PCR Positive n (%)	Real-Time PCR Negative n (%)
Culture-positive TB	28	27 (96.4)	1 (3.6)
Clinical TB (FFPE)	10	5 (50)	5 (50)
Clinical TB (Biopsy)	40	13 (32.5)	27 (67.5)
Non-TB illness	24	0 (0)	24 (100)

PCR: Polymerase chain reaction. FFPE: Formalin-fixed paraffin-embedded.

DISCUSSION

Gastrointestinal TB is often underrated, and yet, any kind of delay in prompt initiation of treatment may lead to treatment failure or to antibiotic resistance development. As the diverse features of the disease in the intestine do not readily suggest a particular diagnosis, diagnostic delays lead to significant morbidity and mortality (2).

A number of rapid investigative methods have been surfacing to aid in the diagnosis of gastrointestinal TB employing diverse *M. tuberculosis* genomic targets, including the IS 6110 insertion sequences (14-16). Undeniably, the PCR systems developed so far have shown good levels of sensitivity (90 to 100%) only on AFB smear-positive samples (17). Therefore, their use has been confined only to when *M. tuberculosis*-specific primers are used for quicker identification of *M. tuberculosis,* hence allowing clinicians to make therapeutic decisions only in the acute phase of infection, i.e. meningitis.

However, conventional "gold standard" AFB smears and in-house PCR tests, though representing suitable strategies, do not yield a confirmatory diagnosis in the histological tissue sections. Further, sole amplification of IS6110 repeat element, the most commonly utilized PCR targets of *M. tuberculosis,* does not allow specific identification of *M. tuberculosis* (18). It has also been found that some strains lack this IS6110 element (19), thereby explaining the reduced sensitivity of in-house PCR (20).

In addition, application of these long-established techniques to FFPE tissues is not without limitations, including DNA structural changes due to prolonged formalin fixation and cross-linking of the DNA (21). Due to the smaller size of the submitted histologic specimens, fully developed histologic features of TB, such as well-formed granuloma with caseous necrosis, are infrequently seen (22). It has also been suggested that the tissue specimens with healed tuberculous granulomas, which were culture- and AFB stain-negative for MTB, would sometimes be positive for MTB DNA (23). Thus, when MTB DNA is found in tissue specimens, a further evaluation of other laboratory and clinical information becomes mandatory, especially before a final diagnosis of TB is made. In this regard, we found that all the patients who reported positive for MTB DNA by LC PCR responded positively to standard ATT-$(HERZ)_2(HR)_{10}$ therapy.

As molecular detection techniques are gaining routine prominence for diagnosis and clinical monitoring of *M. tuberculosis* infection, we were able to show in our study that by using a real-time LC PCR assay, MTBC infection in the intestinal surgically resected tissue sections could be detected with a higher analytical sensitivity than the in-house PCR. An important feature of this real-time PCR assay is that quantification of the *M. tuberculosis* load is obtained by measuring the specific amplification of a 159 bp region of the mycobacterial genome. In addition, the assay utilized a secondary heterologous amplification system in the form of internal control to identify possible PCR inhibition with an analytical detection limit of 1.6 copies/µl (8).

Results of our investigation demonstrated that the real-time detection technology using FRET probes gave a higher positive index. The results obtained for Groups A and B depicted the accuracy of the real-time assay as demonstrated by the congruency in the both qualitative and quantitative results (Table 4). The one negative result obtained in Gro-

Table 3. Diagnostic values of the real-time PCR assay

Test	Category	Diagnostic values Sensitivity %	Specificity %	PPV %	NPV %
PCR (positivity)	Culture-positive TB	96.4	100	100	96
	Clinical TB (FFPE)	50	100	100	83.3
	Clinical TB (Biopsy)	32.5	100	100	96.4
	Total clinical TB	36	100	100	96.9

PCR: Polymerase chain reaction. PPV: Positive predictive value. NPV: Negative predictive value. FFPE: Formalin-fixed paraffin-embedded.

Table 4. Comparison of results of AFB culture / ZN staining and LC PCR of Group C*

Sample type	Site of involvement	Total no. of samples	AFB Culture/ ZN Staining		LightCycler 2.0	
			- ve	+ ve	- ve	+ ve
*FFPE		10	10	-	05	05
	Abdomen	05	05	-	04	01
	Rectum	01	01	-	-	01
	Liver	02	02	-	01	01
	Ileum	02	02	-	-	02
Tissue Biopsy		40	40	-	27	13
	Cecum	10	10	-	05	05
	Colon	18	18	-	17	01
	Ileum	07	07	-	03	04
	Rectum	05	05	-	02	03

FFPE: Formalin-fixed paraffin-embedded tissue. ZN: Ziehl-Neelsen. TB: Tuberculosis. LC PCR: LightCycler polymerase chain reaction. AFP: Acid-fast bacilli

up B may have been due to the uneven distribution of the MTBC in the tissue. The overall positive index of the samples of Group C tested was found to be 36%. The positivity of FFPE and tissue biopsy samples was found to be 50% and 32.5%, respectively (Tables 3, 4). Such highly sensitive real-time LC PCR assay for MTBC DNA confers increased diagnostic accuracy, and this valuable information would add to the overall interpretation of the clinical course of TB monitoring. Our results signify the importance of these molecular assays for better disease management and inconsequently help the patient to be aware of the infection without delay. However, to establish the superiority of this novel technique for the MTBC diagnosis in various latent infectious states, it will be necessary to accumulate data from larger numbers of fractions with suspected TB infection. Above all advances, an integrated approach for the early identification of the disease is the need of the hour.

In conclusion, molecular diagnosis by real-time PCR is useful for early detection of intestinal TB when morphologic features are suggestive but not confirmatory because of negative staining for AFB.

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