Comparision of ERIC-PCR, OUT-PCR and Spoligotyping Methods to Diagnose of Cross-Contamination with *Mycobacterium tuberculosis*

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Abstract

The culture of *Mycobacterium tuberculosis* from clinical specimens is considered to be the gold standard for the diagnosis of tuberculosis. However, false-positive cultures can occur due to cross contamination. Molecular typing methods are a useful tool for quick diagnosis of false-positive culture. In this study, we aimed to comparative evaluation of false-positive culture results by using ERIC-PCR, OUT PCR and spoligotyping methods. In the present investigation, we evaluated a total of 29 culture isolates obtained from five groups samples processed on the same day during 5 different time periods.

By using spoligotyping, 5 spoligotype family including LAM9, LAM7-TUR, T and an unidentified spoligotype were identified according to SITVIT2 database. With the OUT-PCR, 4 different band pattern was determined at Nusieve agarose jel electrophoresis. For ERIC-PCR method, 2-3 band pattern were obtained at Nusive agarose jel and 6-7 band pattern were determined at polyacrylamide gel electrophoresis. All the isolates were collected in two groups by ERIC-PCR, but this created groups were not efficient to determine the degree of separation between isolates. In the study, spoligotyping and OUT-PCR groups modeling were compared with each other revealed compliance rate of 96.55% between the two tests. The application of OUT-PCR has been serve as a rapid and efficient method to investigate cases of possible cross-contamination with *M. tuberculosis*.

Keywords: Cross contamination;ERIC-PCR; OUT-PCR; spoligotyping; *Mycobacterium tuberculosis*

Introduction

The isolation of *Mycobacterium tuberculosis* complex organisms from clinical specimens collected from suspected patients serves as the gold standard for the proper diagnosis of tuberculosis [1]. The cross isolation with culture is the most important step for definitive diagnosis of tuberculosis. False-positive cultures occur due to contaminated clinical equipment, clerical errors or cross-contamination of specimens.

The cross-contamination frequency has been reported between 0.1% to 3% of *M. tuberculosis* in the published studies. Indications of cross-contamination include culture results inconsistent with the patient’s clinical course, unexpected drug resistance, single culture-positive specimens and solid-medium cultures with low-colony-number counts [2-7].

A single positive culture is the most commonly reported indicator of false positivity. False-positive results for tuberculosis have been a matter of concern because of the clinical, therapeutic, and social impacts of the misdiagnosis of tuberculosis. At the same time, these false-positive cultures lead to an overestimation of the incidence and prevalence of tuberculosis in humans [8-10].

The false positivity due to laboratory cross-contamination is suspected when

a. *Mycobacterium tuberculosis* (MTB) is cultured from only one of the serial specimens of a patient
b. the bacterial yield in the culture is low and
c. the suspected sample has been processed together (or
in a short period of time apart) with at least one other from a patient with a high bacterial load.

Molecular genotyping methods are widely used to determine cross-contamination depend on *M. tuberculosis* in the laboratory.

A definitive demonstration of cross-contamination requires the application of molecular tools to prove that the MTB isolates from the co-processed specimens share identical genotypic patterns. IS6110 RFLP, spoligotyping, MIRU-VNTR and MST are the most commonly used molecular methods for cross contamination [11-16]. Within these methods, IS6110 RFLP is accepted as gold standard. But, this method requires well-grown cultures and takes a long time to provide an answer; usually longer than is acceptable for microbiologists and clinicians to make decisions. Moreover, the latter method requires an advanced laboratory equipment and substantial amount of time due to the fastidious nature of *M. tuberculosis*.

More importantly from, a strictly technical perspective, IS6110-RFLP analysis does a poor job of indicating the presence of *M. tuberculosis* when these organisms contain only a few copies of the IS6110 sequence. Recently, PCR-based techniques have proven to be rapid methods for the resolution of cross-contamination events [17-19]. In our study, 29 culture isolates suspected with cross-contamination occurring at five separate periods have been evaluated for the appropriateness of routine applications of two different easy to use PCR methods comparison with spoligotyping in the genotyping of *Mycobacterium tuberculosis*. One of the PCR based typing methods is OUT-PCR that is specific for amplifying IS6110 in using outward direction primer (OUT-primer), other is ERIC-PCR. ERIC (enterobacterial repetitive intergenic consensus) is specific genomic sequence which was found in Gram negative bacterial genom.

### Materials and Methods

#### Table 1: Specimens groups.

| Type of Specimens | Group of Specimens | Total |
|-------------------|--------------------|-------|
|                   | G1                 |       |
|                   | G2                 |       |
|                   | G3                 |       |
|                   | G4                 |       |
|                   | G5                 |       |
| Urine             | 7                  | 1     | 2 | 2 | 12 |
| Sputum            | 3                  | -     | 2 | 1 | 7  |
| Gastric lavage    | 1                  | 3     | - | 1 | 2  |
| Ascites           | 1                  | -     | - | 1 | 2  |
| Synovial fluid    | -                  | -     | - | 1 | 1  |
| Total             | 12                 | 4     | 2 | 4 | 7  | 29 |

In our study, 5 groups specimens (totaly 29 samples) conducted routine laboratory procedures on the same day in 5 separate periods (sample acceptance, smear microscopy, culture) was be evaluated. 29 specimen was consisted of 12 urine, 7 sputum, 7 gastric lavage, 4 ascites and 3 synovial fluid (Table 1). All the applications related to clinical material (smear, culture cultivation and microscopic preparations, etc.) has been performed in a class II biosafety cabinet in P3 laboratory. Clinic material processing, smear microscopy and culture cultivation are carried out in accordance with the standards defined by the World Health Organization [20-22].

DNA extraction from the isolates and the control strains was performed according to solid phase absorption method (QIAamp DNA Mini Kit, Qiagen, Valencia, CA). The DNA extract of each sample was stored at -20 °C. For OUT-PCR; CGG GGG TTC CCG GAC III (I: Inosine) (Iontek, Bursa) single primer enabled to DNA amplification at outer region of IS6110 was used [17,19]. The PCR reaction was subjected to 40 cycles of amplification (3 min at 94 °C, 1 min at 94 °C, 1 min at 62 °C ); this was followed by 1 min of extension at 72 °C.

For ERIC-PCR; ERIC1R(Tb-2) 5’ATG TAA GCT CCT GGG GAT GAC III (I: Inosine) (İontek-Bursa) primer pairs were used. The composition of the PCR mixture (50μl ) was 5μl 10x Taq PCR buffer (500mM KCl, 100mM Tris-HCL, pH:8.3), Master AmpTM, 5μl 10x PCR Enhancer (Epicenter, Technologies), 3μl MgCl2 (25mM), 5μl dNTP(mix-2mM), 0.75μl ERIC1R (87pmol/μl) primer, 0.9μl ERIC2 (73pmol/μl) primer and Taq polimerase (2U) and 1μl template DNA. The PCR reaction was performed to 35 cycles of amplification (2 min at 94 oC, 45 sec at 94 oC, 1 min at 52 oC 1 min at 52 oC) and 20 min at 72 oC final extension. The presence of amplified product was confirmed by 1.4% NuSieve agarose gel (Sigma, St Louis, MO, USA) [23].

For DNA band analysis; UVI soft,UVI band, Windows Application V99.06 (BioRad,England) programme was used. Four main groups revealed by OUT-PCR method. Four main group was detected in OUT-PCR. The samples which 100% compatible each other were collected in the same group, genotypically, (Figure 1).

With the ERIC-PCR, 2-3 band pattern was detected on NuSieve agarose gel electrophoresis for all isolates. For better distinction, polyacrylamide gel electrophoresis was performed. According to this test results, 6-7 bant pattern was obtained. The isolates were collected in two group according to two electrophoresisresults.

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However, these groups were not enough to determine the degree of separation between isolates (Figure 2).

The spoligotyping was carried out with a commercial kit (Isogen Bioscience BV, Maarsen, The Netherlands) according to the manufacturer’s instructions. The 43 spacers between the direct repeats in the target region were amplified by using DRA biotinylated at the 5’ end and DBR primers. The PCR product was hybridized to a membrane containing 43 oligonucleotides derived from the spacer sequences of \textit{M. tuberculosis} H37Rv and \textit{M. bovis} BCG P3 by reverse line blotting. \textit{M. tuberculosis} H37Rv and \textit{M. bovis} BCG P3 were used as control for spoligotyping. Spoligotypes results were converted into octal code and in the SITVIT2 proprietary database of the Pasteur Institute of Guadeloupe, which is an updated version of the previously released SpolDB4 database.

At the time of the present study, the database contained genotyping information on about 67,000 \textit{M. tuberculosis} clinical isolates from 160 countries of origin. In this database, spoligotype international type (SIT) designates a spoligotyping pattern shared by two or more patient isolates. Major spoligotyping-based phylogenetic clades were assigned according to signatures provided in SpolDB4 [24] (Figure 3).

**Sample group**

The evaluations of the 3 microscopic samples carried out in successive days, shows that 10 clinical samples (sputum, urine, gastric lavage fluid) out of 12 were negative. A sample (acid liquid) evaluated in one day had the negative microscopy result. Of these samples thought to be cross-contamination were determined less than 8 colony in the culture tubes. The clinical results of all the samples were negative. In another sample processed in the same day, the microscopy was three positive (3+) and in the culture tubes two positive (2+) reproduction was observed.

The patient had clinically active \textit{tuberculosis}. When these groups OUT-PCR band patterns and spoligotypes were evaluated in themselves, among 12 samples in a sample, which had positive microscopy and culture results, different band patterns were detected. The band patterns of other 11 samples were the same. 11 samples belonged to spoligotype LAM9 family and in one sample a different spoligotype belonging to T2 family was detected. In OUT PCR and spoligotyping methods 100% compatibility was detected (Table 2).

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**Table 2**: Test results of the first sample group.

| Sample Group | Test Results |
|--------------|-------------|
| Microscopy   | Culture     | Spoligotype pattern | OUT PCR pattern | Clinic |
| 11 samples   | -           | 6-8 colony          | LAM9            | OP1    | -     |
| 1 sample     | +++         | ++                  | T2              | OP2    | Active TB |

**Sample group**

4 samples processed in the same day were negative. In this group the contamination cause couldn't be defined. In the second sample group, 3 samples out of 4 had negative microscopic and clinical results. In the culture tubes of these samples a reproduction which is less than 5 colonies, was observed. The microscopy result of one sample had a negative result and 6-8 colonies were detected in its culture. This sample was clinically positive at the same time. With OUT-PCR method the same band pattern was detected in all 4 samples. The spoligotype of all samples were identified as T1. In OUT PCR ve spoligotipleme methods 100% compatibility was detected (Table 3).

**Table 3**: Test results of the second sample group.

| Sample Group | Test Results |
|--------------|-------------|
| Microscopy   | Culture     | Spoligotype pattern | OUT PCR pattern | Clinic |
| 3 samples    | -           | 2-4 colony         | T1              | OP2    | -     |
| 1 sample     | +           | 6-10 colony        | T1              | OP2    | Active TB |

**Sample group**

The microscopic results of two samples in the third sample group were defined negative and in the culture tubes two colonies were observed. The OUT-PCR band pattern and spoligotype of these samples were the same. In OUT PCR ve spoligotipleme methods 100% compatibility was detected. It was detected that the contamination was resulted from the positive sample (Table 4).

**Table 4**: Test results of third sample group.

| Sample Group | Test Results |
|--------------|-------------|
| Microscopy   | Culture     | Spoligotype pattern | OUT PCR pattern | Clinic |
| 1 sample     | -           | 2 colony            | T               | OP2    | -     |
| 1 sample     | +           | ++                  | T               | OP2    | Active TB |

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Sample group

2 out of 4 samples in the fourth group had negative microscopic results and in their culture tubes 7-8 colony were observed. The patients were clinically negative. The microscopic results of the other 2 samples were 1+ positive. The microscopic results of both was 1 positive. Among two Kültür pozitifiği detected samples, one belonged to a patient who had been receiving tuberculosis treatment and the other belonged to a new tuberculosis patient. Two of the samples belonged to T1 spoligotype family but unidentified spoligotype was detected in two samples. In OUT PCR ve spoligotipleme methods 100% compatibility was detected (Table 5).

Table 5: Test results of fourth sample group.

| Sample Group | Test Results |
|--------------|--------------|
| Microscopy   | Culture      | Spoligotype pattern | OUT PCR pattern | Clinic          |
| 2 sample     | -            | 7-8 colony          | T1               | OP2             | -               |
| 1 sample     | 1+           | 4 colony            | ?                | OP3             | Active TB       |
| 1 sample     | 1+           | 1+                  | ?                | OP3             | Active TB       |

Sample group

Table 6: Test results of the fifth group.

| Sample Group | Test Results |
|--------------|--------------|
| Microscopy   | Culture      | Spoligotype pattern | OUT PCR pattern | Spoligotype Clinic |
| 5 samples    | -            | 2-9 colony          | LAM7-TUR        | OP4              | -               |
| 1 sample     | -            | 2-9 colony          | LAM7-TUR        | 1OP1             |                 |
| 1 sample     | +            | 6-10 colony         | T               | 1OP2             | Active TB under treatment |

5 out of 7 samples in the fifth group had negative microscopic results and in their culture tubes 2-9 colonies were observed. Other 2 samples were detected as microscopically positive, one of them was 3 positive and the other was 1 positive. The culture of these samples were 1-4 positive. The two samples which were detected as culture positive were both belonged to patients who were undergoing tuberculosis treatment. In the OUT-PCR result the same band pattern was detected in 6 samples but one sample had a different band pattern. It was determined that 6 samples belonged to LAM7-TUR family and 1 belonged to T family. In 5 samples belonging to LAM7-TUR family the same OUT-PCR pattern was detected but one sample had a different pattern. In the 5th group between OUT PCR ve spoligotipleme methods 85.71% compatibility was detected (Table 6).

Discussion

The molecular typing methods have been used as an important indicator in the tuberculosis laboratories in determining the source of transmission in cases where there is a doubt of cross-contamination [2-5]. The most important expectation for these methods is the determination of some similar genotypes which cause a false increase in the group and the identification of the epidemiological relationship [11,12,24]. Despite the acceptance of IS6110-RFLP as a primary standard, the need for high quantity of pure DNA, the long duration of the test procedure, the complexity of the process, the high amount of the workload, and the necessity for advanced computer softwares for the analysis of the restriction pieces restrains the area of routine use.

Also with this test the typing of the origins which have less copies than six and the IS6110-RFLP profiles are similar is not adequate [12]. On the other hand Spoligotyping is known as a cheaper, faster, easier and highly sensitive method. For marked oligonucleotide absorbed membranes the evaluation of the test results in the method which is based on the hybridization of PCR product, doesn't require computer hardware [24].

The expected results with these methods are the high distinction capacity between the origins, repeatability and easy application. In the study during 5 different routine application time, the cross contamination possibility of the clinical samples processed in the same day, PZR based ERIC PCR, OUT-PCR and spoligotyping methods are relatively evaluated. In 5 sample groups which were assumed to have cross contamination, with OUT-PCR method 4, with spoligotiplendirme 6 different band patterns were detected out of 29 samples. Since the ERIC-PCR method is not standardized it couldn't be evaluated. 96.55% compatibility was detected between spoligotyping and OUT-PCR group modelling tests when they were compared with eachother.

When the demographic and clinical data of the samples in all groups were analysed, it was detected that there were
no epidemiological relation between the patients. The ERIC-PCR method was found inadequate in terms of its difficulty in standardization and lack of capacity to provide enough distinction, on the other hand OUT-PCR method was determined to be adequate by means of its easy application and its ability to show the distinctions during the typing of the isolates which are few in number.

To determine the proximity degree of a large number of isolates, the use of a program is necessary. It was detected that OUT-PCR method is very sufficient for the distinction of different origins unlike ERIC-PCR method. As a result, it is determined that OUT-PCR method is very efficient for the detection of laboratory cross contaminations in terms of being practical and fast but can not be used alone as method in epidemiological studies.

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