

Full Length Research Paper

Efficacy of infected tissue sample brief-culture on Lowenstein-Jensen media as pre-polymerase chain reaction (Pre-PCR) to diagnosis of *Mycobacterium*

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***Mycobacterium Tuberculosis* (Mtb), the causative agent of Tuberculosis does not retain any bacteriological stain due to the high lipid content in its wall. Classic methods like acid-fast staining or Ziehl-Neelsen staining are used for the diagnosis. Nucleic acid amplification techniques such as PCR are very useful in the rapid diagnosis of infections by *M. tuberculosis* but the sensitivity of polymerase chain reaction PCR is considerably lower because of the presence of inhibitory substances in tissues. The aim of this study was to improve the sensitivity of PCR in inhibiting tissue samples. Thirty-six lymph nodes isolated from inoculated guinea pigs with BCG vaccine. After grinding and suspending samples in sterile distilled water; they were cultured on Lowenstein-Jensen. DNA extraction had been done after 5 days by cetyltrimethylammonium bromide (CTAB) method. Finally, PCR had been done for each extracted DNA. Sensitivity of PCR before culture was 27.8% but 62.5% following brief-culture. The sensitivity elevated by short term culture on LJ and then PCR. It is due to diffusion of inhibitory substances into the substrate, as well as to increase the number of bacteria after the brief-culture.**

Key words: *Mycobacteria*, tuberculosis, polymerase chain reaction (PCR), brief-culture.

INTRODUCTION

With increasing number of infectious diseases by *Mycobacterium tuberculosis* and other *Mycobacteria*, also as per centers for disease control and prevention (CDC) report in 1999, infective dose (ID₅₀) of *Mycobacterium tuberculosis* for human is less than 10 bacilli, thus consideration to rapid identification and differentiation is necessary. Some techniques to detection are Ziehl-Neelsen staining, culture on solid and liquid media, polymerase chain reaction (PCR) and follow of it restriction fragment length polymorphism, and repetitive elements of bacterial DNA like variable number tandem repeats (VNTRs) and so on (Li et al., 2009; Cernicchiaro

et al., 2008).

Detection of acid-fast bacilli (AFB) by smear and Ziehl-Neelsen staining is simple, sensitive and rapid. For instance, AFB would be detected when sputum sample has between 5×10^3 to 5×10^4 AFB/ml (Pfyffer and Vincent, 2005). European Society for Microbiology in 1991 reported that 60% of smear would be positive, if 10^4 AFB/ml are present. Although, according to studies, overall sensitivity range of smear with enough bacteria in specimens is 20 to 80% (Pfyffer and Vincent, 2005; Goodvin, 2007). Lowenstein-Jensen media create optimal conditions to neutralize clinical specimens' toxic compounds. A few of the organisms as 10^1 to 10^2 viable AFB/ml is necessary for getting best result. Therefore, culture is more sensitive than smear (Pfyffer and Vincent, 2005).

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Table 1. Results from PCR before and after brief culture.

Result	PCR	
	Before culture	After culture
Positive	10	25
Negative	26	11

Molecular methods have presented enormous progress in accuracy and rapidity diagnosis in microbiology world; especially diagnostic of fastidious and slow growing bacteria like *M. tuberculosis* (Ortu et al., 2006). PCR as one of the molecular methods is a good method to be used in many laboratories (Honore et al., 2003; Boddinhous et al., 2001). But sometimes, it occurs decreasing in sensitivity when working on clinical specimens such as sputum, bronchial aspirates, bronchoalveolar lavage, pleural, tissues, gastric aspirates biopsy specimens and so on. Because these samples have inhibitory compounds that interfere with PCR process (Pfyffer and Vincent, 2005).

Hence, we decided on using PCR following a brief culture (partial culture) and evaluating the effect of it on PCR sensitivity. By this manner, our expectation was the elevation of PCR sensitivity after neutralizing inhibitory elements in samples by culturing on Lowenstein- Jensen media, as far as possible.

MATERIALS AND METHODS

Sample inoculation

Forty guinea pigs were inoculated in their peritoneum with BCG vaccine (4 guinea pigs were lost sooner than 3 to 4 weeks; therefore we omitted them from this study). After 3 to 4 weeks, lymph nodes were isolated and sent to PIRC LAB (Pasteur Institute Research Centre Laboratory in IRAN). All samples were mixed and homogenized with sterile distilled water (if some had been paraffinized, the first it was deparaffinized) (Bandera et al., 1998; Ozkara et al., 1998; Marchetti et al., 1998).

Sample processing

All specimens were decontaminated by the standard protocol. Two milliliters of the homogenate was separated for decontamination by a modification of the method proposed by Tacquet and Tison (1961) by the addition of 3 ml of a solution of 1% sodium hydroxide 3% lauryl sulfate, after which they were incubated for 30 min. The mixture was neutralized with 8.75% orthophosphoric acid by using bromocresol blue as an indicator, and the neutralized suspension was centrifuged at 3,500 rpm for 30 min (Lie'bana et al., 1995). After re-suspension, two Lowenstein-Jensen slants were considered for each specimen. They were inoculated at 37°C for 5 days. Smears were stained with Ziehl-Neelsen (ZN) staining.

DNA extraction

After 5 days, Lowenstein-Jensen surfaces was washed with 1 ml of

Tris-EDTA, pH 7.6 and added to 9 ml Tris-EDTA (TE), pH 7.6 in falcon tube. Tube was incubated for 20 min in 80°C to inactivation bacteria. Sample was centrifuged at 4500 xg for 10 min, and the supernatant was discarded. After vortex with 1 ml TE, added 9 ml TE and above processes repeated for 3 times. 35 µl of 10% sodium dodecyl sulfate was added to 500 µl samples. They were vortexed for a few seconds and incubated for 10 min at 65°C. The samples were incubated in a solution of CTAB-NaCl for 10 min at 65°C and then mixed with an equal volume of chloroformisoamyl alcohol and centrifuged for 15 min at 14,000 rpm. The aqueous phase (650 µl) was then separated and mixed with an equal volume of isopropanol. The samples were left at -20°C for 30 min and then centrifuged for 15 min at 13,000 x g. The DNA pellet was washed once with 70% ethanol, air dried, and resuspended in lysis buffer and neutralizing reagent (v/v), final volume was 100 µl (Honore et al., 2003).

PCR

The total reaction volume in the first PCR round was 50 ml, and the reaction mixture consisted of 250 mM for each dNTP, 1 mM MgCl₂, 1.25 U of *Taq* DNA polymerase, 5 ml of 10x *Taq* polymerase buffer (supplied with the enzyme), and primers (IS1: 5'-CCTGCGAGCGTAGGCGTCGG-3' and IS2: 5'-CTCGTCCAGCGCCGCTTCGG-3') (Ozkara et al., 1998; Marchetti et al., 1998; Purohit et al., 2007) at 0.1 mM each. The total reaction volume in the second round was 50 ml and contained *Taq* polymerase buffer (supplied with the enzyme), each dNTP at a concentration of 125 mM, 1.5 mM MgCl₂, 1.25 U of *Taq* polymerase, and primers IS1 and IS2 at 0.3 mM each. Twenty microliters of a DNA sample was added to the reaction mixture in the first round. Both PCR rounds were conducted with an initial 4-min denaturation step at 94°C coupled to a repeating cycle of 1.5 min at 94°C, 1.5 min at 63°C, and 1.5 min at 72°C for 20 (first round) and 40 (second round) cycles, followed by 7 min of final extension at 72°C. A total of 2.5 ml of the first-round PCR product was transferred to the second-round PCR mixture. The amplified products were visualized by UV transillumination (Marchetti et al., 1998).

RESULTS AND DISCUSSION

In this study, we had forty guinea pigs that four of them died before 3 to 4 week, 36 lymph nodes were sent to PRIC lab. 36 samples were positive in culture and ZN stain. All specimens assessed by two methods; doing PCR before and after 5 days culture. Positive PCR were 27.8% (10 samples) and 69.4% (25 samples) for before and after brief culture, respectively (Table 1). Sensitivity of this modified method was 62.5% and specificity was 100%. Comparison results, showed significant difference

between them ($P < 0.001$). It seems brief culture and then PCR is more reliable than PCR lonely.

DISCUSSION

It is important to select most relevant method in aspect of sensitivity, simplicity and accuracy to diagnosis of tuberculosis. The PCR has acquired importance in the clinical diagnosis of *M. tuberculosis*, and is now an important support in the clinical diagnosis of TB in developed countries (Brown et al., 1999). Despite its considerable demands for technical skills and equipment, it can also be performed successfully in reference laboratories in developing countries (De Francesco et al., 1996). In our strategy CTAB-PCR method was selected because its sensitivity (53.8%) was higher than the other methods (Honore et al., 2003). In study of Mathur et al. (2009), it was found that recovered 68 of 70 (97.1%) isolates of *M. tuberculosis* (Mtb) on LJ medium as compared to 66 (94.2%) by blood agar. The difference was not significant. But mean time to detect macroscopic colonies of Mtb on blood agar was 13.6 ± 5.2 days as compared to 20.4 ± 5.1 days on LJ medium ($p = 0.0001$) (Mathur et al., 2009). More colonies were observed on blood agar than on LJ medium. In our study, LJ was our selected medium. If we used blood agar, might be gain better results after 5 days culture. But we cultured Mtb on LJ because it is standard medium for this bacterium.

In Hernandez et al. (1997) study PCR sensitivity was 85.2% and in Bergmann study it was 94.1%. PCR in both studies had been done regardless kind of samples (different samples were used) (Hernandez et al., 1997; Bergmann and Woods et al., 1999). However, sensitivity of PCR after brief culture (62.5%) in our study was less than these studies; but it showed brief culture significantly had been increased positive results. It seems one reason for difference between these studies and ours, concern to kind of sample. The sensitivity of *M. tuberculosis* PCR (MTB-PCR) is considerably low for tissue samples. It depends on both lower bacterial content and presence of inhibitory substances in tissues.

Several previous studies have evaluated the possibility of rapid detection of MTBC by Bactec vials and PCR. In the majority of these, the commercial AccuProbes were used; the reported sensitivities ranged from 33 to 83% (Body et al., 1990; Reisner et al., 1994; Telenti et al., 1994). A commercial amplification assay (Amplicor MTB; Roche Molecular Systems, Somerville, NJ, USA) has also been investigated for the same purpose; the sensitivities obtained ranged from 66 to 100% (Lie'bana et al., 1995; Purohit et al., 2007; Brown et al., 1999). The Bactec radiometric method is one of the fastest and most sensitive cultural tools for the diagnosis of mycobacterial infections (Pfyffer et al., 1997; Roggenkamp et al., 1999). Wang identified *Mycobacterium tuberculosis* complex in BACTEC cultures by ligase chain reaction, sensitivity in

this study was 99.7% (Wang and Tay et al., 2002). The sensitivity of the radiometric assay allows a culture to reach a positive threshold in a very short time, often within 1 to 2 weeks. Sensitivity test in our study was 62.5% that was between or near range of above results. As it was said, these results had been gained by commercial kits.

Conclusion

In summary, the aim of this study was to demonstrate the importance of optimal DNA extraction and brief culture for elevating efficacy and accuracy PCR for the rapid detection of lymph nodes tuberculosis.

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