

Comparison Between Real-Time Polymerase Chain Reaction and DNA-Microarray in Detection and Identification of Mycobacterium Species

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ABSTRACT. Low-cost and low-density (LCD) DNA arrays offer an easy way to detect resistance as minimal laboratory instrumentation is needed. Nucleic acid-based amplification tests allow the rapid detection of Mycobacterium tuberculosis. Recently, a real-time PCR assay for M. tuberculosis complex was introduced. Real-Time PCR and DNA-microarray techniques were compared with the classical methods of Ziehl-Neelsen (ZN) staining and culturing. Regarding to the standard culture method, 80 positive individuals were identified out of 140 urine samples. RT-PCR showed 96.3% sensitivity and 96.7% specificity with Mycobacterial tuberculosis complex (MTB) (n=10) and nontuberculous mycobacteria (NTM) (n=70). The DNA-microarray analysis exhibited 100% sensitivity and specificity. One species belonging to (MTB) was identified as M. tuberculosis and positively represented by 12.5% (n=10). Five species belonging to nontuberculous mycobacteria (NTM) were identified and represented as M. kansasii 37.5% (n=30), M. celatum 21.25% (n=17), M. gordonae 11.25% (n=9), M. chelonae 10% (n=8), and M. phlei 7.5% (n=6). The results recommend the use of our simple and rapid PCR technique for early diagnosis of mycobacteria's.

Also, the fast LCD-microarray protocol is very useful for species identification and differentiation between MTB and NTM.

KEY WORDS: Polymerase Chain Reaction, Mycobacterium Species, DNA-microarray

INTRODUCTION

Tuberculosis remains a worldwide public health problem. Its causative agent is *Mycobacterium tuberculosis* which causes death in developing countries (Martens G, 2007). Non-tuberculous mycobacteria (NTM) are responsible for most of mycobacterial infections in resource-rich countries, where tuberculosis is not endemic (Glassroth J (2008). Furthermore, infections with NTM have increased mainly in patients with a compromised immune system (Wolinsky, E, Katoch, VM., Paolo JR (2004). Mixed infections with *M. tuberculosis* and NTM have been also reported (Libanore, M, 1992).

With the recent global issue of mycobacterial infections, it is necessary to find rapid, sensitive, and specific diagnostic methods for the detection and identification of *M. tuberculosis* and NTM in clinics (Bloom BR, Murray, CJL. 1992). The traditional diagnosis of mycobacterial infection is accomplished by a culture-based identification method. It is a time consuming due to its slow growth which usually takes 4 to 6 weeks or longer (Jeager H, 1967). Direct examination with microscope is faster but it lacks sensitivity and specificity. Rapid and sensitive diagnosis not only prevents of resistance and further spread of infections but also avoids unnecessary drug exposure (Griffith, DE, 2007)

Recent methodological advances in molecular biology have provided alternative rapid approaches for detection or identification of *M. tuberculosis*. These include the PCR and PCR-linked methods (Eisenach, KD, Eing, BR, Sechi, LA, Suffys, P, Montenegro, SH) The choice of the target sequence, which should be present in all tuberculosis mycobacteria and absent in all other bacteria, is crucial for the successful detection (Del Portillo (1996), P Mustafa, AS (1999). These targets included 16S and 23S rRNA genes (Fries J (1990), Patel, J. B (2000). the *hsp65* genes coding for 65-kDa heat shock protein Hance, A.J (1989), Pao, C.C (1990) the *dnaJ* gene [Takewaki, SI 1993) the *gyrB* gene Kasai, H (2003). and IS6110 characteristic insertion sequences for mycobacterium Thierry, D (1990), Ross, BC (1993), Shawar, RM (1993).

Differentiation between *M. tuberculosis* and other members of the *Mycobacterium* species is very important for control of the disease and prescription of an appropriate and effective treatment. Recently, multiplex PCR and microarray techniques were used successfully for rapid detection, identification, and differentiation between the *Mycobacterium* species. Fukushima M (2003), Park H (2005), Tolber N (2006), Kee S (2009).

The present study is an attempt to assess the role of PCR in the diagnosis of tuberculosis in comparison with other conventional methods. This was performed by screening 140 patients, suspected for tuberculosis, using two primers derived from conservative regions of mycobacterial 16S rDNA gene sequence. Furthermore, the study extended to identify the species of the mycobacterium isolates, either *M. tuberculosis* or *Mycobacterium* other than tuberculosis using a rapid diagnosis of microarray technique.

MATERIALS AND METHODS

Sample preparation, staining and culturing

A total of 140 urine samples from patients (86 male and 54 female) with suspected tuberculosis (i.e. clinical symptoms, urinalysis and radiographic finding) were collected during 2009, from the Urology and Nephrology Center, Mansoura University, Egypt. All samples were coded and processed at the time of collection according to the standard methods of decontamination with N-acetyl-L-cysteine-NaOH Robert, GD (1991). Each urine specimen was then concentrated by centrifugation at 4000 rpm for 30 min. The centrifuged deposits were pooled and divided into aliquots for Ziehl-Neelsen staining, culturing, and PCR detection. A routine direct microscopic examination using Ziehl-Neelsen Acid Fast staining protocol was performed according to Hindler and Munro Hindler, FJ (2007). An aliquot was used for TB culture by Versa TREK (ESP Culture System II-Trek Diagnostic Systems, Inc., Westlake, Ohio). The cultures were automatically monitored every 24hr depending on the rate of oxygen consumption within the head space of the culture bottle.

Bacterial strains

The bacterial strains used for testing the sensitivity of the designed primers for the 16S rRNA other than Mycobacterium species were obtained from Urology and Nephrology Center, Mansoura University and listed as follow:

| Strain | Features |
|-------------------------------|---------------|
| <i>Escherichia coli</i> | Gram Negative |
| <i>Pseudomonas aeruginosa</i> | Gram Negative |
| <i>Staphylococcus aureus</i> | Gram Positive |
| <i>Enterococcus faecalis</i> | Gram Positive |

DNA extraction

DNA was extracted from the aliquots of the samples centrifuged deposits using High Pure Template Purification Kit (Roch, Germany). The purified chromosomal DNA was used for PCR and microarray analysis.

Polymerase chain reaction

The primers were designed depending on the DNA sequence alignments of the mycobacterial 16S rRNA conservative regions. The approximate expected size of the amplified DNA target is 1350 bp. The following forward and reverse primers were manufactured in the laboratories of The Midland Certified Reagent Company Inc. of Midland, Texas.

MTB-F: (5`-GAGTTTGATCCTGGCTCAGGA-3`) Tm = 54.3°C.

MTB-R: (5`-GCTTCGGGTGTTACCGACTT-3`) Tm = 53.8°C.

DNA amplification was carried out in a final volume of 50 µl, with 0.4 µM of each primer, 200 µM of each dNTPs, 10 mM Tris HCl, pH 8.3, 50 mM KCl; 0.1 % triton X-100, 1 U of Taq-polymerase (Pharmacia) and 5 µl of the prepared DNA extract from clinical samples. After initial denaturation at 96°C for 10 minutes, 45 amplification cycles were performed within automated Thermal Cycler (Applied Biosystem 9700). Each cycle consisted of denaturation at 94°C for 2 min., annealing of primers at 50°C for 2 minutes and primer extension at 72°C for 3 min., increasing each extension every cycle by 3 seconds.

DNA electrophoresis

The amplified DNA product was resolved on 1.4 % agarose gel containing 0.5 mg/ml ethidium bromide and visualized by UV transilluminator.

Microarray analysis

LCD array structure and design

The LCD array kit (Myc^{Direct} 1.7) was manufactured by Chipron GmbH, (Germany) and designed for identification of *Mycobacterium tuberculosis* complex and other Nontuberculous mycobacteria (NTM). It is based on a PCR amplification of rRNA gene region with 225-265 bp depending on species, and a 126 bp fragment from the IS6110 element. The previous two DNA targets of PCR products were allowed to hybridize with immobilize DNA probes which were designed depending on available data base entries of mycobacterial species. Each LCD-chip contains eight identical microarrays in rectangular reaction chambers which can be addressed individually. The formed array is an 8 x 8 pattern with average spot diameter of 300 µm. the capture probes are immobilized as duplicates (vertical) and the functional controls are located in three angles.

Amplification, hybridization and reading

The PCR for microarray was performed in a 25 µl volume. 115 µl was prepared for 5 reactions by adding 5 µl of each two sets of primer mixes (A and/or B) provided with the array kit, 5 µl of dNTPs 10 mM each, 12.5 µl of 10x buffer included 25mM MgCl₂, 1.5 µl of 5U/µl Taq polymerase, and the final volume of 115 µl was completed with DNase free water. The mixture was aliquot in 5 PCR tube then 2µl of extracted bacterial DNA were added to each tube. The Thermal cycler setting was adjusted for 3 minutes at 96°C for initial denaturation, followed by 35 cycles each one contained 20 seconds at 94°C for denaturation, 30 seconds at 58°C for primers annealing, and 30 seconds at 72°C for extension. Additional 3 minutes at 72°C for final extension were performed.

Microarray hybridization was performed according to the provided protocol of the LCD array kit (Myco^{Direct} 1.7). The LCD chip was scanned using Chip Scanner PF 2700 and the data was analyzed by SlideReader V7.00.01 software provided from Chipron GmbH.

Statistical analysis

The percentages of sensitivity, specificity and efficiency were calculated according to the following formulas (Zhang et al., 1999):

$$\% \text{ sensitivity} = \text{true positives} / (\text{true positives} + \text{false negative}) \times 100.$$

$$\% \text{ specificity} = \text{true negative} / (\text{true negative} + \text{false positive}) \times 100.$$

$$\% \text{ efficiency} = (\text{true positive} + \text{true negative}) / \text{number of samples} \times 100.$$

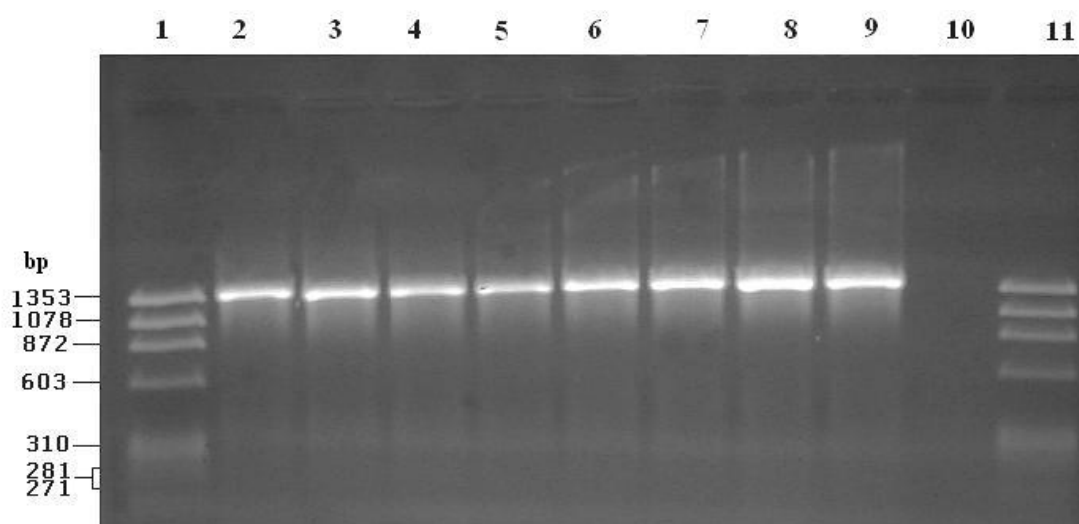
RESULTS

Out of 140 urine samples (86 male and 54 female) from patients suspected with tuberculosis, only 80 individuals (31 male and 49 female) gave positive mycobacterial culture test. On the other hand, Ziehl-Neelsen staining test showed only 54 individuals (23 male and 31 female) with positive results (Table 1).

Table 1 Distribution of Ziehl-Neelsen and Culture Positive cases among 140 samples suspected for *Mycobacterium*.

| | Patients No. | Microscopy (ZN) | | | Culture | | |
|--------|--------------|-----------------|-----|--------|---------|-----|--------|
| | | +ve | -ve | +ve % | +ve | -ve | +ve % |
| Mal | 45 | 23 | 31 | 42.5 % | 31 | 23 | 57.4 % |
| Female | 86 | 31 | 55 | 36.0 % | 49 | 37 | 57.0 % |
| Total | 140 | 54 | 86 | 38.6 % | 80 | 60 | 57.1 % |

The two primers MTB-F and MTB-R had been used for *Mycobacterium* detection from the previous samples by using PCR method. The resulting PCR product revealed the expected size of about 1350 bp DNA fragment of 16S rRNA gene for all the *Mycobacterium* species (Figure 1).



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Figure 1. The PCR products of about 1350 bp from Mycobacterium species using the 16S rRNA gene based MTB-F and MTB-R primers. Lanes 2 and 3 for *M. tuberculosis*; lanes 4 and 5 for *M. kansasii*; and lanes 6-9 for *M. goodnae*, *M. phlei*, *M. chelonae* and *M. celatum* respectively. Lane 10 is control. Lanes 1 and 11 are DNA marker.

Furthermore, no specific PCR product was detected when some other Gram positive and negative bacterial strains had been tested by the same two primers at the same PCR conditions (Data not shown). The comparison between the PCR and ZN methods regarding to the standard culture method for *Mycobacterium* detection was represented in Table 2.

Table 2. Detection of *Mycobacterium* in 140 samples by PCR of 16S rRNA gene compared with ZN, and Culture methods.

| Microscopy (ZN) | PCR | Number of samples in terms of culture result | | |
|-----------------|--------------|--|-----------|------------|
| | | Positive | Negative | Total |
| Positive | Positive | 54 | 0 | 54 |
| | Negative | 0 | 0 | 0 |
| | Total | 54 | 0 | 54 |
| Negative | Positive | 23 | 2 | 25 |
| | Negative | 3 | 58 | 61 |
| | Total | 26 | 60 | 86 |
| Total | Positive | 77 | 2 | 79 |
| | Negative | 3 | 58 | 61 |
| | Total | 80 | 60 | 140 |

Out of the 80 culture positive samples, only 77 exhibited PCR true positive results. While 2 false positive PCR results were obtained from the 60 culture negative samples. All the positive ZN tests (54 samples) gave also positive results for PCR and culture. On the other hand, 23 false negative ZN samples exhibited positive PCR and culture results. In addition, 3 false negative ZN and PCR showed positive culture detection. The calculated percentages of sensitivity, specificity and efficiency for the ZN and PCR methods are represented in Table 3. PCR technique showed 96.3% sensitivity comparing with ZN which gave only 67.5%. Contrary, ZN showed 100% of specificity while the PCR exhibited 96.7%. The calculated efficiency was 81.4% and 96.4% for ZN and PCR detection respectively.

Table 3. Comparison between the microscopy (ZN) and PCR of 16S rRNA gene detection of *Mycobacterium* within the 80 culture positive samples out of 140.

| Reading | ZN | PCR |
|-----------------|---------------|---------------|
| True Positive | 54 | 77 |
| False Positive | 0 | 2 |
| True Negative | 60 | 58 |
| False Negative | 26 | 3 |
| Sensitivity (%) | 67.5 % | 96.3 % |
| Specificity (%) | 100 | 96.7 % |
| Efficiency (%) | 81.4 % | 96.4 % |

The LCD array analysis showed that the 80 positive cultures from urine specimens contained six species of pathogenic mycobacterial isolates. The LCD array assay showed different patterns according to the *Mycobacterium* species (Figure 2). One species belonging to Mycobacterial tuberculosis complex (MTB) was identified as *Mycobacterium tuberculosis* and positively represented by 12.5% (n=10) out of the 80 positive samples. Five species (within the 70 positive culture sample) of nontuberculous mycobacteria (NTM) were identified and represented as *Mycobacterium kansasii* 37.5% (n=30), *Mycobacterium celatum* 21.25% (n=17), *Mycobacterium goodnae* 11.25% (n=9), *Mycobacterium chelonae* 10% (n=8), and *Mycobacterium phlei* 7.5% (n=6) as shown in Table (4).

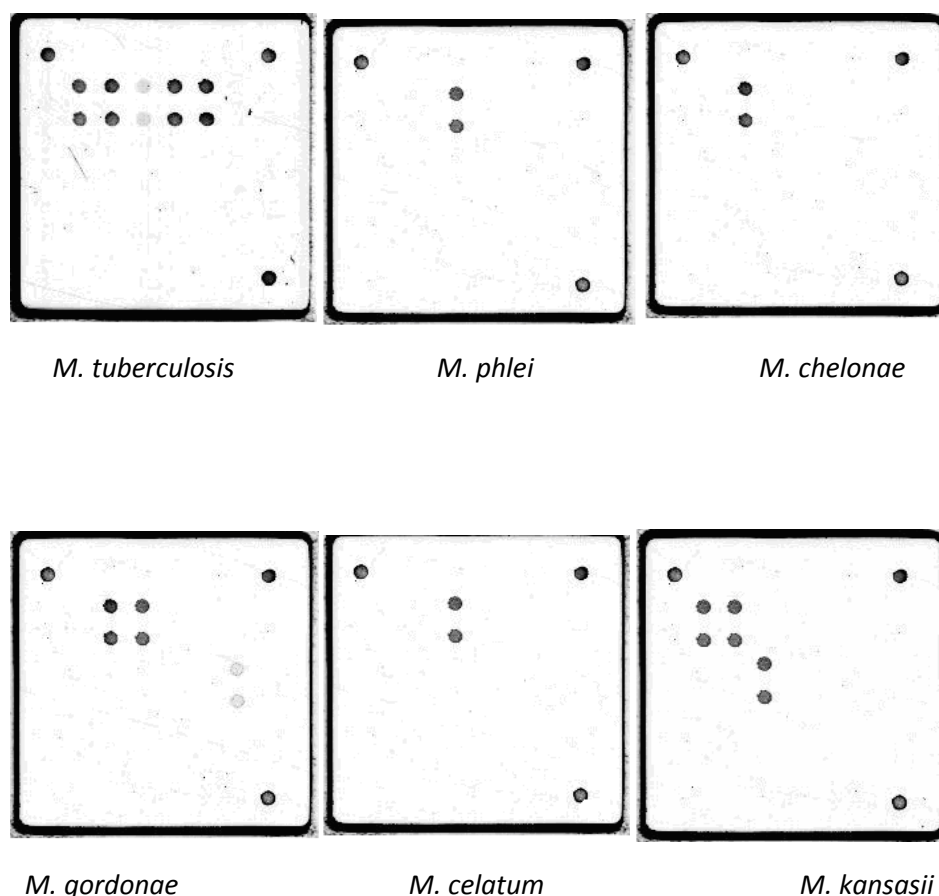


Figure 2. The LCD-microarray patterns of the different *Mycobacterium* species.

Table 4. Identification of the *Mycobacterium* species as detected by LCD-array for the 80 positive culture samples.

| Type | Strain | No. of +ve LCD array | +ve % |
|-------|------------------------|----------------------|---------|
| MTB | <i>M. tuberculosis</i> | 10 | 12.5 % |
| NTM | <i>M. kansasii</i> | 30 | 37.5 % |
| | <i>M. celatum</i> | 17 | 21.25 % |
| | <i>M. gordonae</i> | 9 | 11.25 % |
| | <i>M. chelonae</i> | 8 | 10 % |
| | <i>M. phlei</i> | 6 | 7.5 % |
| Total | | 80 | |

The PCR products resulted in array assay using only primer mix (A) showed also different pattern and approximate expected sizes of DNA fragments depending on each species of *Mycobacterium* (Figure 3). Primer mix (B) produced about 126 bp for all culture positive samples (Figure 4).

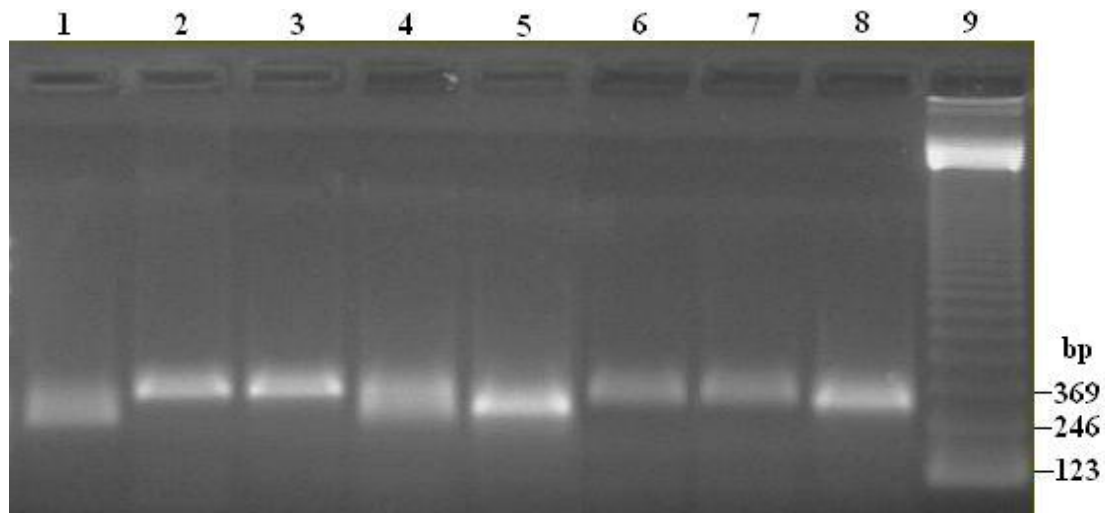


Figure 3. PCR assay using primer mix A for differentiation of *Mycobacterium tuberculosis* complex (MTB) and nontuberculous mycobacteria (NTM). The size of the PCR products is depending on species-specificity. *M. gordonae* (216 bp) lane 1; *M. phlei* (311bp ~313 bp) lanes 2 and 3; *M. kansasii* (229 bp) lane 4; *M. tuberculosis* (226 bp) lane5; *M. chelonae* (262 bp) lanes 6 and 7; *M. celatum* (234 bp) lane 8; DNA marker lane 9.

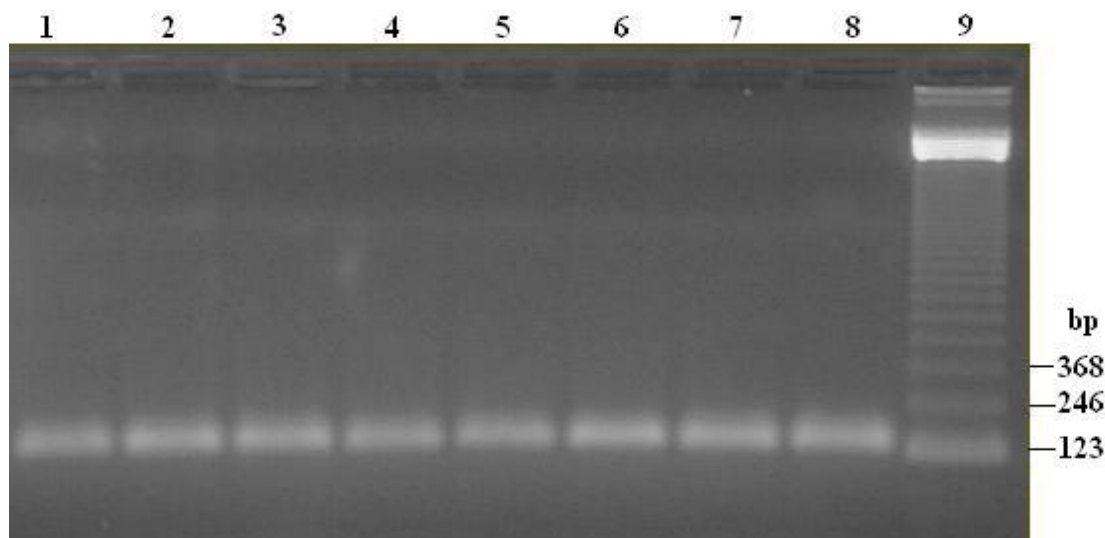


Figure 4. PCR assay using primer mix B which produced specific (126 bp) bands for both *Mycobacterium tuberculosis* complex (MTB) and nontuberculous mycobacteria (NTM). *M. gordonae* lane 1; *M. phlei* lanes 2 and 3; *M. kansasii* lane 4; *M. tuberculosis* lane5; *M. chelonae* lanes 6 and 7; *M. celatum* lane 8; DNA marker is lane 9.

DISCUSSION

Mycobacterium tuberculosis has been found in various body fluids such as sputum, urine, pus and lymph node aspirates. Here we examined 140 urine samples suspected of having tuberculosis using traditional (ZN and culture) and molecular (PCR and LCD-microarray) tools. Only 80 individuals (57.1%) were detected as culture positive with 100% of specificity. When conventional method of ZN staining was used, only 54 individuals (38.5%) were positive. Using the culture as the reference test, ZN sensitivity and specificity reached 67.5% and 100% respectively. ZN smear examination has been previously reported to have sensitivity of 33.79% (Negi et al.), 41% (Aderaye et al. 2007), and 65.4% (El-Dawi et al. 2004). Much higher sensitivity for ZN microscopic smear examination was recorded which ranging from 84% (WHO) to 88% (Shinnick and Jonas 1994). In spite of the high specificity of ZN staining method, it showed variable and low sensitivity which is mainly attributed to the degree of mycobacterium shedding in a sample. Although the culture remains the standard method for the definitive diagnosis of tuberculosis, it is a time consuming. Nucleic acid amplification methods to detect *M. tuberculosis* are increasingly in use as a rapid and accurate diagnostic tool. This study aimed to assess the value of using a PCR method in the routine diagnosis of either MTB or NTM. Our PCR technique based on amplification

of about 1.35 Kb DNA fragment from the mycobacterial 16S rRNA genes, which are highly stable rather than insertion elements which are likely to undergo deletions Radhakrishnan, I (2001). The PCR result exhibited 96.3% sensitivity and 96.7% specificity, with three false negative and two false positive samples. The presence of false-negative results can be due to insufficient number of samples or the presence of inhibitors Barouni, AS (2004). Although the MTB-F and MTB-R primers did not show any DNA amplification for some Gram negative and positive bacteria, they produced two false positive results. This might be attributed to the specimen's contamination during the handling of samples. Richardson et al. (2009) used multiplex real-time PCR primers targeting the 16s rRNA gene and the internal transcribed spacer (ITSs) for identification of 314 mycobacterial culture positive samples. Their results showed about 99% sensitivity and specificity for *M. tuberculosis* complex, *M. avium* complex, *M. chelonae-M. abscessus-M. immunogenum* group and *M. mucogenicum*. The same targets exhibited 95% sensitivity and 100% specificity for *M. fortuitum* group (MFG). Slightly lower sensitivity and specificity, reached 94.5% and 95.9% respectively, were obtained using another multiprimer depending on the IS6110 insertion element, the genus specific fragment (32kDa) and species-specific *mtp40* gene (Barouni, A. S 2004)

Microarray-based species identification for *Mycobacterium* is a widely used technology. The LCD-array used in this study was based on two mixes of multiprimers, which amplify 216-313 bp of 16SrRNA depending on *Mycobacterium* species and a 126 bp fragment from the IS6110 element. The results showed 100% of sensitivity and specificity for MTB or NTM when LCD-microarray was applied. Furthermore, 37.5% and 21.25% of the true positive mycobacterium infections were due to *M. kansasii* and *M. celatum* respectively. This agrees with Richardson, ET 2009, Marras, TK, 2002) who reported that *M. kansasii* is more common for other geographical regions.

In conclusion, the simple one step PCR using MTM-F and MTB-R primers based on the 16S rRNA exhibited high sensitivity and specificity would give an encouragement for MTB and NTM rapid diagnosis. Also, it is recommended to use the LCD-microarray for the mycobacterial species identification as a rapid and highly sensitive diagnosis rather than classical methods. This would help the clinics to prescribe suitable and convenient anti-TB drugs or even shorten unnecessary antibiotic exposure for the non-tuberculosis patients.

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