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ORIGINAL ARTICLE

Detection and identification of *Mycobacterium* species

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ABSTRACT

Background: *Successful diagnosis and effective treatment for mycobacterial infections are mainly depending on a rapid and sensitive identification method.* ***Objective:*** *To detect and identify the Mycobacterium species.* ***Methodology:*** *PCR and LCD-microarry techniques were compared with the classical methods of Ziehl-Neelsen staining (ZN) and culturing. Two primers based on two conservative regions within the mycobacterium 16S rRNA gene were designed and amplified a DNA fragment of about 1350 bp for both complex of Mycobacterium tuberculosis (MTB) and non-tuberculous mycobacteria (NTM).* ***Results:*** *Regarding to the standard method of culture, 57 positive individuals were identified out of 100 urine samples. The PCR showed 96.30 % sensitivity and 96.70% specificity, while ZN gave Se = 67.50 % and Sp = 100 %. The LCD-microarray analysis exhibited 100 % sensitivity and specificity. One species of MTB was determined as M. tuberculosis and positively represented by 12.3% (n=7). Five species of NTM were determined and represented as M. kansasii 36.8 % (n=21), M. celatum 21 % (n=12), M. gordonae 12.2% (n=7), M. chelonae 10.5 % (n=6), and M. phlei 7% (n=4).* ***Conclusion:*** *The results recommended utilizing the simple and rapid PCR method for early mycobacteria detection. Also, the fast LCD-microarray protocol is very beneficial for identification and differentiation between MTB and of NTM species.*

INTRODUCTION

Tuberculosis (TB) remains a public health concern worldwide. Its causative agent is *Mycobacterium tuberculosis* (MTB), which causes death in developing countries[1]. Nontuberculous mycobacteria (NTM) are responsible for the majority of infections by mycobacterial in resource-rich countries, where tuberculosis is not endemic[2]. In addition, NTM infections have primarily risen in patients suffering from a compromised immune system[3,4,5]. Mixed infections with *MTB* and *NTM* have been also reported[6].

With the recent global issue of mycobacterial infections, it is necessarily to find rapid, sensitive, and specific diagnostic methods for detection and identification of *MTB* and *NTM* in clinics[7,8]. The traditional diagnosis of infection by mycobacteria species is achieved by means of a culture-based identification method. It is a time consuming due to its slow growth which usually takes 4 to 6 weeks or longer[9]. Direct examination with microscope is faster but it lacks sensitivity (Se) and specificity (Sp). Rapid and sensitive diagnosis not only prevents resistance and further spreading of infections but also avoids unnecessary drug exposure [10].

Recent methodological advances in molecular biology provide alternative rapid approaches for detection or identification of *M. tuberculosis*. These include the PCR and PCR-linked methods [11- 15]. The choice of the target sequence that must be present in all MTB species and absent in all other bacterial strains, is crucial for an effective detection [16, 17]. These targets included 16S and 23S rRNA genes, the *hsp65* genes coding for 65-kDa heat shock protein, *dnaJ* gene, *gyrB* gene, and IS*6110* characteristic insertion sequences for mycobacterium [18-21].

Differentiation between *M.tuberculosis* and other members of *Mycobacterium* strains is very important for control of infection and prescription of an appropriate and effective treatment. Multiplex PCR and microarray techniques have recently been successfully used to rapidly diagnose, identify and differentiate between Mycobacterium organisms [22-25].

The present research is an attempt to assess the role of PCR in the *TB* diagnosis in comparison with other conventional methods. Furthermore, the current research extended to identify the species of the mycobacterium isolates, either *M. tuberculosis* or *Mycobacterium* other than *TB* using a rapid diagnosis of microarray technique.

METHODOLOGY

Sample preparation, staining and culturing:

A total of 100 urine samples were collected from routine work of Microbiology Laboratory in Urology and nephrology center, Mansoura University from April 2011 to November 2011. All samples were coded and processed at the time of collection according to the standard techniques of decontamination with N-acetyl-

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L-cysteine-NaOH[26]. 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 made according to[27]. An aliquot was used for TB culture by VersaTREK (ESP Culture System II-Trek Diagnostic Systems, Inc., Westlake, Ohio). The cultures were automatically monitored every 24 hrs depending on rate of oxygen consumption within the head space of culture bottle.

DNA extraction

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

MTB-F: (5`-GAGTTTGATCCTGGCTCAGGA-3`) Tm = 54.3^{o}C.

MTB-R: (5`-GCTTCGGGTGTTACCGACTT-3`) Tm = 53.8^{o}C.

DNA amplification was made in a final volume of 50 µl, with 0.4 µM of each primer, 200 µM of each dNTPs, 10 mM Tris HC1, pH 8.3, 50 mM KC1; 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 involved denaturation step at 94°C for 2 min., annealing of primers at 50°C for 2 min. and primer extension at 72°C for 3 min., increasing each extension every cycle by 3 seconds.

Microarray analysis

LCD array structure and desiegn

LCD array kit (Myco ^{Direct} 1.7) was manufactured by Chipron GmbH, (Germany) and designed for identification of complex of MTB and other 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 IS*6110* 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 chip of LCD has eight identical microarrays in rectangular reaction chambers that can be individually handled. 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

PCR for microarray was accomplished 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 $MgCl_2$, 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 all tubes. The Thermal cycler setting was adjusted for 3 minutes at 96^{o}C for initial denaturation, followed by 35 cycles each one contained 20 seconds at 94^{o}C for denaturation, 30 seconds at 58^{o}C for primers annealing, and 30 seconds at 72^{o}C for extension. Additional 3 minutes at 72^{o}C for final extension were performed.

According to the provided LCD array kit protocol (Myco Direct 1.7), microarray hybridization was performed. Using Chip Scanner PF 2700, the LCD chip was scanned and the data was analysed by Chipron GmbH's SlideReader V7.00.01 programme.

DNA electrophoresis

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

Statistical analysis:

The percentages of sensitivity, specificity and efficiency were estimated according to Zhang et al.[28]

RESULTS

Out of 100 urine samples collected, only 57 individuals gave positive mycobacterial culture test. Whereas, Ziehl-Neelsen staining test showed only 39 individuals with positive results (Table 1).

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

Group	Samples No.	Miroscopy (ZN)			Culture		
		+ve	-ve	+ve %	+ve	-ve	+ve %
Total	100	**39**	61	39 %	**57**	43	57 %

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 *Mycobacterium* strains (Figures 1, 2, and 3).

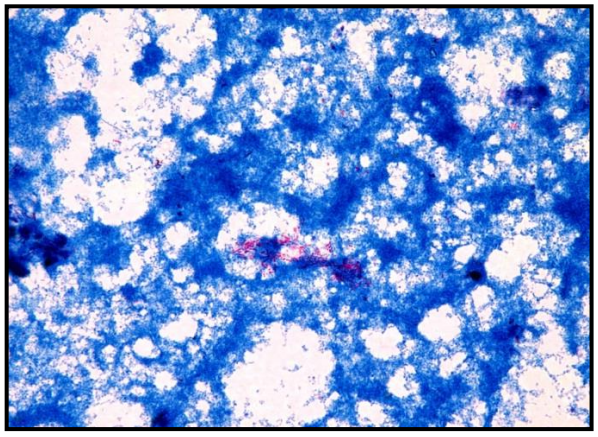

Fig. 1: Acid fast bacilli staining appears in red color indicating positive results for urine sample. The blue color is a counter stain. (Magnification 1000 X)

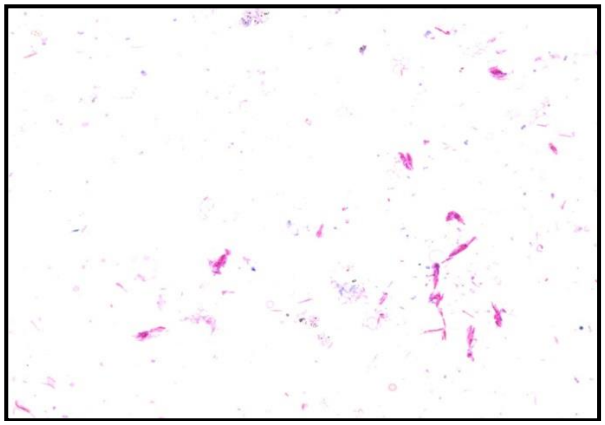

Fig. 2: Acid fast bacilli staining appears in red color indicating positive results for cultures. (Magnification 1000 X)

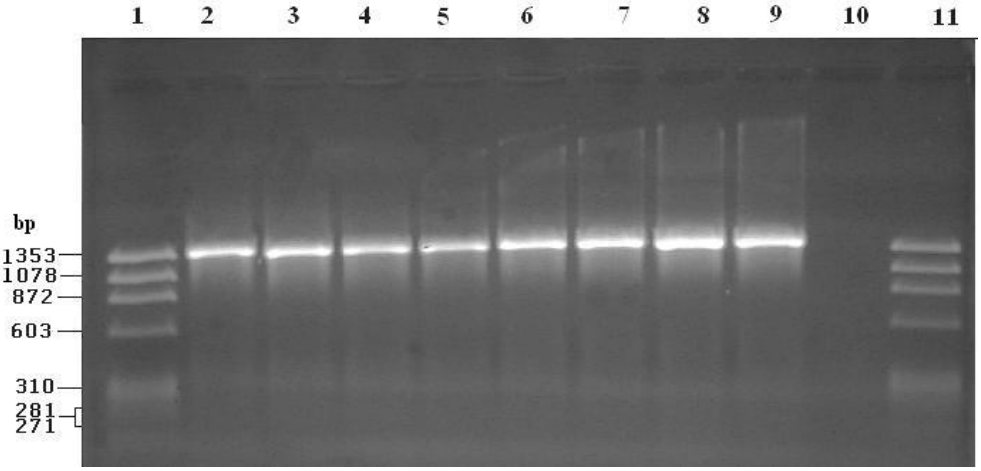

Fig. 3: The PCR products of about 1350 bp from Mycobacterium species using 16S rRNA gene based MTB-F and MTB-R primers. Lane-2 and Lane-3 for *M. tuberculosis*; lanes 4 and 5 for *M. kansasii*; and lanes 6-9 for *M. gordonae, M. phlei, M. chelonae* and *M. celatum,* respectively. Lane-10 is control.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

The comparison between the PCR and ZN methods regarding to standard culture method for *Mycobacterium* detection was represented in (Table 2). Out of the 57 culture positive samples, only 55 exhibited PCR true positive results. While 2 false positive PCR results were obtained from the 43 culture negative samples. All the positive ZN tests (39 samples) gave also positive results for PCR and culture. Alternatively, 16 false negative ZN samples exhibited positive PCR and culture results. In addition, 2 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 81.40 % and 96.40 % for ZN and PCR detection respectively (Tables 2, 3).

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	39	0	39
	Negative	0	0	0
	Total	39	0	39
Negative	Positive	16	2	18
	Negative	2	41	43
	Total	18	43	61
Total	Positive	55	2	57
	Negative	2	41	43
	Total	57	43	100

Table 3: Comparison between (ZN) and PCR of 16S rRNA

Reading	ZN	PCR
True Positive	39	55
False Positive	0	2
True Negative	42	41
False Negative	19	2
Sensitivity (%)	67.5 %	96.3 %
Specificity (%)	100	96.7 %
Efficiency (%)	81.4 %	96.4 %

The LCD array analysis showed that 57 positive cultures from urine specimens contained six species of pathogenic mycobacterial isolates. LCD array assay showed different patterns according to *Mycobacterium* strains (Figure 4). One strain belonging to complex of Mycobacterial tuberculosis (MTB) was determined as *Mycobacterium tuberculosis* and positively displayed by 12.50 % (n=7) out of the 57 positive samples. Five species (within the 50 positive culture sample) of NTM were recognized and represented as *Mycobacterium kansasii* 36.8 % (n=21), *Mycobacterium celatum* 21 % (n=12), *Mycobacterium gordonae* 11.2% (n=7), *Mycobacterium chelonae* 10.5% (n=6), and *Mycobacterium phlei* 7% (n=4) as displayed in Table (4), (figure 4).

Table 4: Identification of *Mycobacterium* species as detected by LCD-array

Type	Strain	No. of +ve LCD array	+ve %
MTB	*M. tuberculosis*	7	12.2 %
NTM	*M. kansasii*	21	37 %
	M. celatum	12	21 %
	M. gordonae	7	12.2 %
	M. chelonae	6	10.5 %
	M. phlei	4	7 %
Total		**57**	

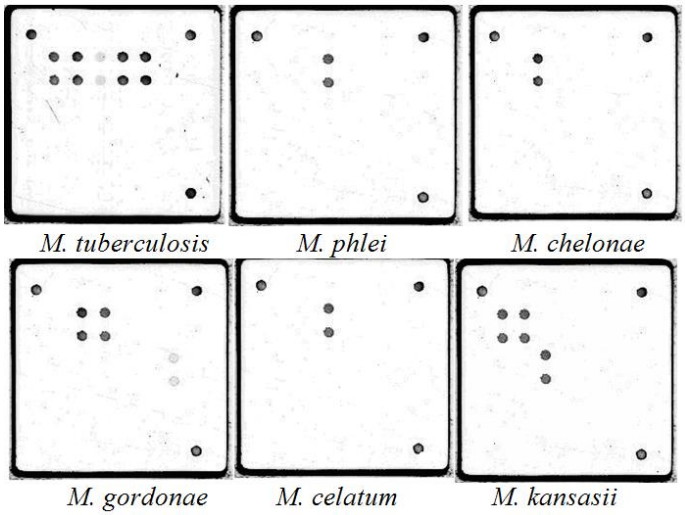

Fig. 4: LCD-microarray patterns of the different *Mycobacterium* species.

The PCR products resulted in array assay utilizing only primer mix (A) showed also different pattern and approximate expected sizes of DNA fragments depending on each species of *Mycobacterium*. Primer mix (B) produced about 126 bp for all culture positive samples (Figure 5), (Figure 6).

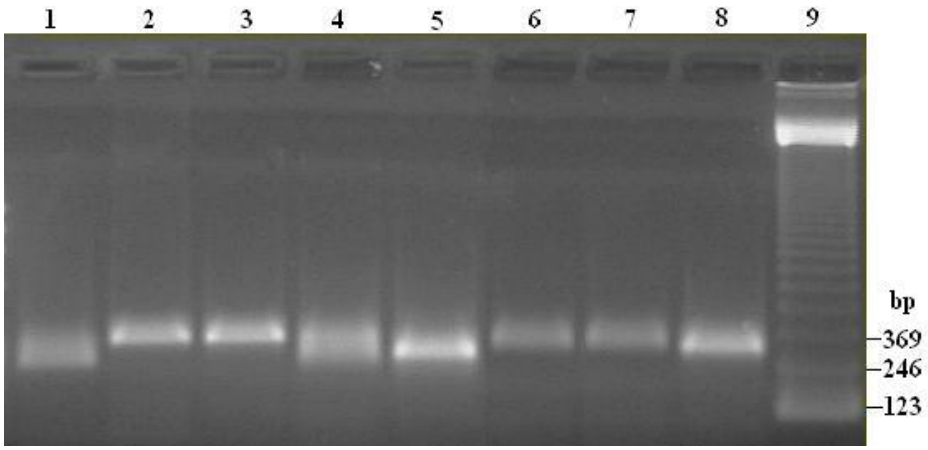

Fig. 5: PCR assay using primer mix A for differentiation of MTB complex and NTM. The size of the PCR products is depending on species-specificity. *M. gordonae* (216 bp) lane 1; *M. phlei* (311bp ~313 bp) lane- 2 and Lane- 3; *M. kansasii* (229 bp) lane 4; *M. tuberculosis* (226 bp) lane5; *M. chelonae* (262 bp*)* lanes 6 and 7; *M. celatum* (234 bp) lane 8; DNA marker lane 9.

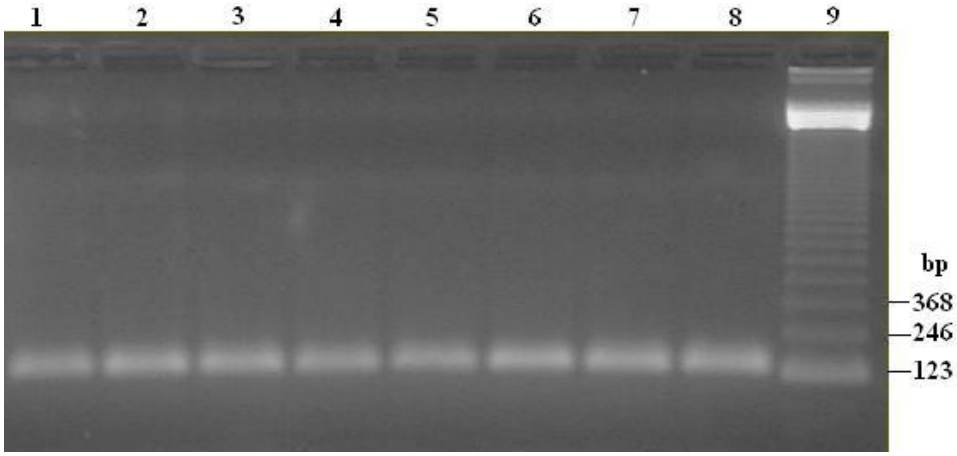

Figure (6): PCR assay using primer mix B which produced specific (126 bp) bands for both MTB complex and NTM. *M. gordonae* lane 1; *M. phlei* lane- 2 and Lane-3 ; *M. kansasii* lane 4; *M. tuberculosis* lane5; *M. chelonae* lanes 6 and 7; *M. celatum* lane 8; DNA marker is lane 9.

DISCUSSION

Mycobacterium tuberculosis has been found in various body fluids such as sputum, urine, pus and lymph node aspirates. Out of 100 urine samples examined for tuberculosis using traditional (ZN and culture) and molecular (PCR and LCD-microarray) tools, only 57 samples (57%) were detected as culture positive with 100% of specificity. When conventional method of ZN staining was used, only 39 samples (39%) 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 % [29], 41.00%[30], and 65.40%[31]. Much higher sensitivity for ZN microscopic smear examination was recorded, which ranges from 84.0%[32] to 88% [33]. In spite of high specificity of ZN 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 typical method for definitive TB diagnosis, it is a time consuming.

Methods of Nucleic Acid amplification for detection of *M. Tuberculosis* is increasingly being used as a fast and reliable method for diagnosis. 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 [34]. The PCR result showed SE= 96.30 % and SP= 96.70 %, with three false negative and two false positive specimens.

The presence of false-negative results can be attributable to insufficient amount of samples or the presence of inhibitors [35]. 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.[36] used multiplex real-time PCR primers targeting 16s rRNA gene and the internal transcribed spacer (ITSs) for identification of 314 mucobacterial 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 SE&SP, reached 94.5% and 95.9% respectively, were obtained using another multiprimer depending on the IS*6110* insertion element, the genus specific fragment (32kDa) and species-specific *mtp*40 gene [35].

Microarray-based species identification for *Mycobacterium* is a widely used technology[22-24]. 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 IS*6110* element. The results showed 100% of SE&SP for MTB or NTM when LCD-microarray was applied. Furthermore, 37.50 % and 21.25 % of true positive mycobacterium infections were attributable to *M. kansasii* and *M. celatum* , respectively. This agrees with Marras and Daley [37] who reported that *M. kansasii* is more common for other geographical regions.

CONCLUSION

The simple one step PCR using MTM-F and MTB-R primers based on 16S rRNA exhibited high SE&SP 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.

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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