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SENSITIVITY AND SPECIFICITY OF CONVENTIONAL CULTURE, LIGHT MICROSCOPY, SEROLOGICAL AND MOLECULAR METHODS FOR IDENTIFYING *Mycobacterium tuberculosis* COMPLEX

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ABSTRACT

In this study, some different tools, *i.e.*, conventional culture, light microscopy, serological and molecular methods were evaluated for their sensitivities to detect the *Mycobacterium tuberculosis* complex in forty specimens from animal and human sources. To achieve such goal 20 samples from each of raw milk, animal tissues, and human CSF were collected. Enzyme-linked immunosorbent assay (ELISA) was compared to conventional culture, light microscopy for its sensitivity and specificity to detect the presence of TB pathogen in these samples. Results showed that sensitivity and specificity of 10% &100% for IS6110-targed PCR, 30 and 90% for ELISA, 16.25% and 100% for conventional culturing and 17.5% and 87.0% for light microscopy were recorded. In other mean, the IS6110-targed PCR was relatively more useful in TB diagnosis followed by ELISA. Furthermore, the DNA fingerprinting of three standards TB strains using random amplified polymorphic of DNA-PCR (RAPD-PCR) was carried out in the hope of generating some DNA molecular marker for TB identification. RAPD-PCR finding showed some molecular DNA markers that could be very useful in the identification of tuberculosis strains.

Key Words: *M. tuberculosis* complex, TB diagnosis, Light microscopy, ELISA, PCR, DNA fingerprinting, RAPD-PCR.

INTRODUCTION

It worth to mention that tuberculosis was one of the most common infectious diseases in the world, as it has been recorded as an important causal agent of the death of between adults throughout the world (Cosivi *et al*., 1998 and Travería *et al*., 2013). Shinnick *et al*., (1995) estimated that one third of the world human's populations were infected with *Mycobacterium tuberculosis* and other *Mycobacterium* species. The etiological agent of bovine tuberculosis was *M. bovis*, which classified as a member of the TB complex and causing tuberculosis in humans as a result of its presence in pasteurized milk that considered an important part of a person's diet (Sherris, 1984).

Yeager *et al*., (1967) showed that light microscopy of acid-fast microorganisms lacks sensitivity and can only detect bacteria in concentration of 10000/mL or greater. Polymerase chain reaction (PCR) including the IS6110 insertion sequence, has been described by Hawkey (1994) and Collyns et al., (2002). This sequence was detected in multiple copies in the genome of M. tuberculosis, M. bovis, M. *africanum* and *M. microti* which belonging to the *M. tuberculosis* complex (Brissson-Noel *et al*., 1989, Eisenach *et al*., 1990 and Collins *et al*., 1993).

Sampaio *et al*., (2006) applied four molecular typing methods for analysis of *M. fortuitum* group strains causing post-mammoplasty infections.

This study was designed to evaluate the sensitivity and specificity of different tools, *i.e.*, conventional culture, light microscopy (acid-fast staining), enzyme-linked immunosorbent assay (ELISA) for detecting the presence of TB complex in samples from animal and human resources. A trail to optimize the PCR conditions as well as generating some specific DNA markers for TB identification *via* random amplified polymerphic of DNA-PCR (RAPD-PCR) was also determined.

MATERIALS AND METHODS

It is worth noting that this study was completed as part of an MA thesis in the Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University during the period from 2002 to 2003.

Bacterial strains: The standard mycobacterial strain used in this study was *M. tuberculosis* H37-Ra (B) which obtained from the American Type Culture Collection (ATCC). The mycobacterial strains M0-041 (A) and M03-035 (C) which used for RAPD-PCR analysis were obtained from two different Egyptian patients.

Sources of samples: A number of 20 samples from each of milk and tissue samples (15 from animals suspected to be infected with TB and 5 negative controls) were collected from Fayoum Governorate. Similarly, sample numbers were collected from CSF and sputum from patients in Abasiya Fever Hospital, Cairo, Egypt.

Samples preparation: In case of milk samples, 10 mL aliquots were processed and centrifuged at 4000 rpm for 10 min. All cream and fluid were poured off. A volume of 200 µL were decontaminated by adding 4% sodium hydroxide as described by Kubicia *et al*., (1963). CSF samples were divided into two parts and one of them was centrifuged at 3000 rpm for 15 min. Sputum samples processed according to digestion denomination procedure developed by Kubicia *et al*., (1963).

Light microscopy: The stained smears were microscopically examined as applied by Singh and Parija (1998).

Mycobacteria cultivation: On tube of Lowenstein Jensen and Stone Brink medium two drops of the processed samples were placed then incubated at a slant

with the screw cap loose at 37ºC for a week. Cultures were examined weekly for growth throughout 12 weeks.

ELISA detection: From *M. tuberculosis* H37Ra, the culture filtrate antigen was prepared as described by Nassau *et al*., (1975). For ELISA detection of the presence of antibodies in the CSF samples using a microtiter plate as reported by Kiran *et al*., (1985).

DNA extraction: In this experiment, a suspension of *M. tuberculosis* H37Ra strain was prepared in TE buffer (Sambrook *et al*., 1989), and cooled to room temperature then centrifuged at 4000rpm for 5 min. The supernatant was transferred to a fresh tube and stored at -20ºC. On the other hand, DNA was extracted from tissue, CSF and sputum samples using the QIAamp Blood and Tissue Kit (Qiagen). For milk, 1.0mL of milk was centrifuged at 6000rpm for 10min. The clear whey portion was suctioned out with a transfer pipette and discarded. The remaining milk solids and butterfat were used for further processing and DNA extraction.

Primers used: Two oligonucleotides named P1: 5'CCTGCGACGTAGGCGTCGG3' and P2: 5'CTCGT CCAGCGCCGCTTGG3' originally designed by Eisenach *et al*., (1990) were used as primers.

Optimization of PCR conditions: Different conc-entrations from each of DNA template (25, 50, 100, 150 and 200 ng); dNTPs (100, 150 and 299 µM); primers (0.4, 0.8 and 1.2 µM) and *Taq* DNA poly-merase 1.0, 1.25 and 2.5U were tested to optimize the PCR conditions for detection of TB.

PCR amplification: The reaction mixture of 25 µL containing 10X-PCR buffer, $MgCl_2$, dNTPs, P1 and P2 primers, *Taq* DNA polymerase, DNA template, and sterile distilled water was prepared. The amplification reactions were performed in a GeneAmp PCR System 8799 (Applied Biosystem). The DNA was denaturated for 5 min at 95ºC and then 35 amplification cycles, each consists of 95ºC, 64ºC and 72ºC for 30 sec for each were performed. The last elongation cycle was extracted for 5min. As a negative control, PCR mixture with no template was used.

Gel electrophoresis: In the presence of 50-2000 bp DNA ladder as a marker, the PCR product was electrophoresed in 2% agarose gel in TAE buffer stained with ethidium bromide and visualized under ultraviolet light and photographed as described by Sambrook *et al*., (1989).

Parameters calculation: Calculation of the sensitivity and specificity was determined according to the Manual of Clinical Microbiology (1999) as follows:

$$\% \text{ Sensitivity} = \frac{\text{True positive}}{\text{True positive + false negatives}} \times 100$$

$$\% \text{ Specificity} = \frac{\text{True positives}}{\text{False positive + True negatives}} \times 100$$

RAPD-PCR analysis: The genomic DNA of the three TB strains, *i.e.*, M03-041 (A), H37Ra (B) and M03-035 (C) were subjected to RAPD-PCR analy-sis using sixteen RAPD-PCR oligonucleotides from OPERON Technologies, Alameda, CA, Kits A (3, 6, 9, 10, 11, 16, 17 & 19), B (4, 7, 8, 15 & 20) and C (5,7 and 19). The PCR was carried out and analyzed as described by El-Domyati and Mohamed (2004). A dendrogram using the combined results obtained with the three oligonuc-leotides was constructed.

RESULTS AND DISCUSSION

The use of PCR for detection of *M. tuberculosis* was reported in sputum (Kocagoz *et al*., 1993; Pilkaytis *et al*., 1993; Wilson *et al*., 1993 and Noordhoek *et al*., 1994) and in a routine Mycobacteriology Laboratory (Clarridge III *et al.*, 1993); CSF (Donald *et al*., 1993; Lee *et al*., 1994 and Bonington *et al*., 2000).

For the clinician rapid confirmation of diagnosis of TB continues to be a serious problem, while, the devastating nature of the disease frequently leads to the early detection. This situation has led to the development of number of direct and indirect tools to aid the TB diagnosis. Results in Table-1 showed that percentage of TB detection was varied in 20 samples either from milk, animal tissues, CSF or sputum using the three different tools, *i.e.*, microbiological, PCR, or ELISA. From the tabulated data, it could be recommended that the microbiological tools were preferable than the PCR as a molecular tool; whereas the total percentages of TB detection were ranged from 15.75 to 17.50 in case of the microbiological tools and was 10% when the PCR was used. But in fact, the PCR was higher than the culturing and LM in case of milk and animal tissues and was equal in case of sputum.

Results also showed that ELISA was more effective than PCR in case of CSF, it could be due to the use of antiserum specific to the TB-antibodies *i.e*., detecting the TB *via* indirect way. There-fore, efforts should be done towards producing antiserum against the original microorganism as an antigen(s). In this study the sensitivity of PCR detecting *M. tuberculosis* in CSF samples was also close to that reported for a similar methodology of Shankar *et al*., (1991).

Table 1: Detection of TB in four different samples using microbiological, serological and molecular techniques.

Samples	Microbiological techniques				Serological Technique		Molecular technique	
	Culturing		LM		ELISA		PCR	
	No.	%	No.	%	No.	%	No.	%
Milk	0	0	3	15	*	*	1	10
Tissues	2	10	4	20	*	*	3	15
CSF	8	40	1	10	6	30	1	10
Sputum	3	15	6	30	*	*	3	15
Total	13	16.25	14	17.5	6	30	8	10

*: Not tested. LM: light microscopy. Note: 20 samples were taken from each item.

The experimental results in Table 2 could be considered as a confirmatory study and proved the efficacy of using the recent method such as PCR or ELISA for TB detection. The explanation of this result could be due

to the high cost of culturing equipment and its long required time, which threaten the life of patient. The high specificity of PCR (100%) was obtained within a maximum two days and the lowest cost of ELISA compared with the other three tools.

Mullis and Faloona (1987) showed that PCR was investigated as one of the most sensitive molecular methods for TB detection. Schochetman *et al*., (1988) reported that amplification of specific nucleic acid sequences *via* PCR has become a powerful tool for the rapid and specific detection of different agents. Daniel (1990) showed that in developing countries the rapid diagnosis of TB was too complex for application. Therefore, this study, a trial to optimize the PCR conditions was carried out using different concentrations of each of primer, PCR buffer, $MgCl_2$, dNTPs, DNA as a template and *Taq* DNA polymerase

Table 2: Comparison between four different methods for detection of TB.

Parameters	Microbiological techniques		Serological technique	Molecular technique
	Culturing	LM	ELISA	PCR
Sensitivity (%)	15.75	17.5	30	10
Specificity (%)	100	87	90	100
Required time	3-8 w	2-2.5 h	1-3	1-2 d
Cost (L.E/sample)	19.62	2.16	5.4	12.3

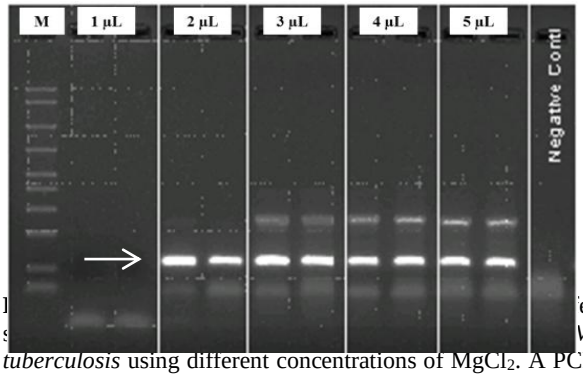
w: Week. h: Hour. d: Day.

Results in Table-3 and Figs. -1-5 showed that 150 mM, 100 µM, 0.4 µM, 25ng and 1 U were the most suitable conditions, respectively for PCR detection of TB. This result indicated the possibility to detect more samples with provided equipment and this will make the cost cheaper. It also could be concluded that, 1) PCR was able to detect very low concentration of the TB. 2) It is possible that a patient, who was negative for a certain pathogen by serological method(s), may be still show symptoms of the disease at later stages caused by that pathogen. The absence of amplified fragments was observed in the negative controls revealed that reaction mixtures were free from any other DNA contamination.

Table 3: Optimization of PCR conditions for TB detection.

PCR mixture components	Concentrations	PCR results
$MgCl_2$ (mM)	100	-
	150	+
	175*	+
	200 *	+
	250 *	+
dNTPs (µM)	100	+
	150	+
	200	+
Primers (µM)	0.4	+
	0.8	+
	1.2	-
Taq DNA polymerase (U)	1.00	+
	1.25	+
	2.50	+
DNA Template (ng)	025	+
	050	+
	100	+
	150	+
	200	+
Negative control		-

-: Negative. +: Positive. *: Nonspecific fragments were amplified.



tuberculosis using different concentrations of $MgCl_2$. A PCR product of 123 bp (Arrow) was amplified. Negative control: PCR mixture without any DNA template. Note, the stock of $MgCl_2$ was 100 mM per µL. M, 50 base DNA marker.

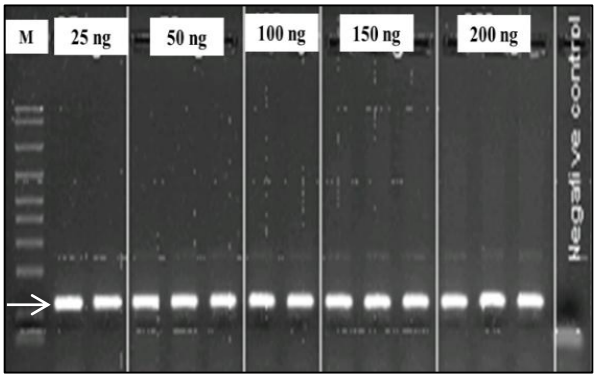


Figure 2: Electrophoresis of agarose gel (1.2%) in TAE buffer stained with ethidium bromide shows detection of *M. tuberculosis* using different concentrations of dNTPs. A PCR product of 123 bp (Arrow) was amplified. Negative control: PCR mixture without any DNA template. M, 50 base DNA marker.

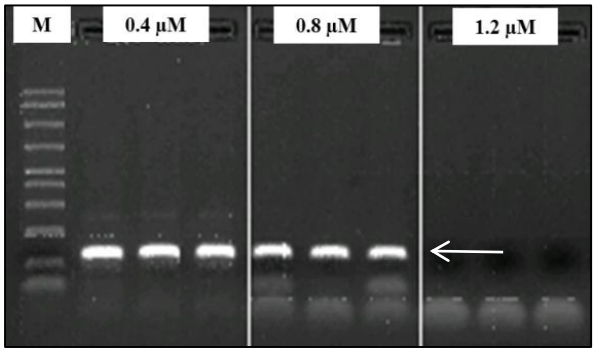


Figure 3: Electrophoresis of agarose gel (1.2%) in TAE buffer stained with ethidium bromide shows detection of *M. tuberculosis* using three different concentrations of primers. A PCR product of 123 bp (Arrow) was amplified. M, 50 base DNA marker.

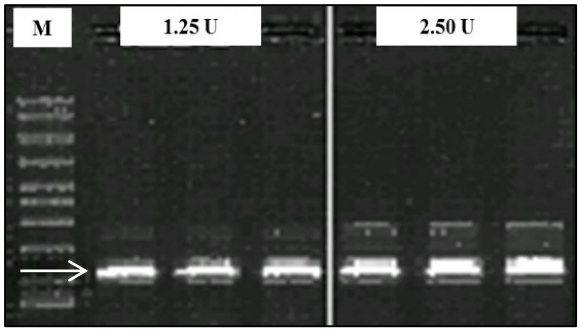


Figure 4: Electrophoresis of agarose gel (1.2%) in TAE buffer stained with ethidium bromide shows detection of *M. tuberculosis* using two concentrations of *Taq* DNA polymerase. A PCR product of 123 bp (Arrow) was amplified. M, 50 base DNA marker.

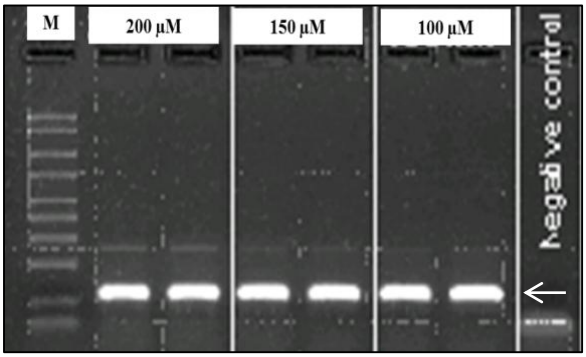


Figure 5: Electrophoresis of agarose gel (1.2%) in TAE buffer stained with ethidium bromide shows detection of *M. tuberculosis* using different concentrations of DNA template. Negative control: PCR mixture without any DNA template. A PCR product of 123 bp (Arrow) was amplified. M, 50 base DNA marker.

Based on a novel DNA sequence Collins *et al*., (2002) used a PCR test for distinguishing some strains of *M. avium* subsp. *Para-tuberculosis* from cattle and sheep. Abu-Amero (2002) paid an attention to the potential for the use of PCR for detection and identification of *M. tuberculosis* complex in sputum.

Williams *et al*., (1990) were the first who des-cribed a new DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence called RAPD. They suggested that these polymerphisms could be named RAPD markers.

Mazurier and Wernars (1992) showed that RAPD was a rapid and simple DNA fingerprinting method. RAPD has been successfully applied to differentiate strains of *M. malmoense* (Kauppinen *et al*., 1994) and *M. tuberculosis* (Linton *et al*., 1994).

De Juan *et al*., (2005) determined the genetic diversity of *M. avium* subspecies *paatuberculosis* isolates from goats detected by pulsed field gel electrophoresis.

Singh *et al*., (2006) investgated the importance of RAPD analysis or typing Indian strains of *M. tuberculosis*. *M. tuberculosis* H37Rv, *M. tuberculosis* DT and 42 clinical isolates of *M. tuberculosis* were subjected to RAPD-PCR using 7 random decamer primers. All 7 primers were found to be differentiated and produced specific RAPD profiles. The polymorphic amplicons served as RAPD markers for *M. tuberculosis.*

In this study RAPD was used to determine the genetic relationship between three strains of TB. Results of RAPD-PCR presented in Tables 4-6 and Figs. 6-7 showed the genetic relationship between the three applied TB strains. For each strain, the number of amplified fragments was differed with different primers (Table 4). Whereas the number of amplified fragments for these strains was ranged from 78 to 96 out of 110 fragments produced using the sixteen primers (Table 5). On the other direction, the number of amplified fragments differed from one strain to another for the same primer. In addition, the strains were characterized by unique fragment(s) with the same used primers (Table 6). In other word, 12, 18, and 15 out of 45 unique fragments were obtained with the TB strains A, B, and C, respectively. However, some bands were common for all strains (Monomorphic bands). Khosravi *et al*., (2015) identified clinical isolates of *M. fortuitum* by RAPD-PCR and ERIC PCR

Table 4: RAPD-PCR analysis of three different strains of *M. tuberculosis* (A, B and C) using their DNA extracts as templates and sixteen RAPD-PCR primers.

DNA amplified fragments		*M. tuberculosis* strains			DNA amplified fragments		*M. tuberculosis* strains		
		M0-041 (A)	H37Ra (B)	M03-035 (C)			M0-041 (A)	H37Ra (B)	M03-035 (C)
OPA03	AMF01	0	0	1	OPB04	AMF05	0	0	1
	AMF02	1	0	0		AMF06	1	0	1
	AMF03	1	0	1	OPB07	AMF01	1	1	1
	AMF04	1	1	1		AMF02	1	0	1
	AMF05	1	1	1		AMF03	1	1	1
	AMF06	1	1	1		AMF04	1	1	1
OPA06	AMF01	0	0	1		AMF05	1	1	1
	AMF02	1	1	1	OPB08	AMF01	1	1	1
	AMF03	1	1	1		AMF02	1	0	0
	AMF04	1	0	0		AMF03	1	1	1
OPA09	AMF01	1	0	1		AMF04	1	1	1
	AMF02	1	1	1		AMF05	1	0	1
	AMF03	1	1	1		AMF06	1	1	1
	AMF04	1	0	0	OPB15	AMF01	1	1	1
	AMF05	1	1	1		AMF02	1	1	1
	AMF06	0	1	0		AMF03	1	0	1
OPA10	AMF01	1	0	1		AMF04	1	1	1
	AMF02	0	0	1		AMF05	1	1	1
	AMF03	1	0	0		AMF06	1	1	1
	AMF04	1	1	1		AMF07	1	1	1
	AMF05	0	1	0		AMF08	1	1	1
	AMF06	1	1	1		AMF09	1	1	1
OPA11	AMF01	1	1	1		AMF10	1	1	1
	AMF02	1	0	1		AMF11	1	1	1
	AMF03	1	1	1	OPB20	AMF01	1	1	1
	AMF04	1	0	0		AMF02	1	1	1
	AMF05	1	1	0		AMF03	1	0	0
	AMF06	1	1	0		AMF04	1	0	0
OPA16	AMF01	1	1	1		AMF05	1	0	0
	AMF02	1	1	1		AMF06	1	0	0
	AMF03	0	0	1		AMF07	1	0	1
OPA17	AMF01	1	1	0		AMF08	1	1	1
	AMF02	1	1	1	OPC05	AMF01	1	1	1
	AMF03	1	1	1		AMF02	1	0	1
	AMF04	1	1	1		AMF03	1	1	1
	AMF05	1	0	0		AMF04	1	1	0
	AMF06	1	0	1		AMF05	0	1	0
	AMF07	1	1	1		AMF06	1	1	0
	AMF08	1	1	1		AMF07	1	1	1
	AMF09	1	0	1	OPC07	AMF01	1	0	1
	AMF10	1	0	1		AMF02	1	1	1
	AMF11	1	1	0		AMF03	1	1	1
OPA19	AMF01	1	1	1		AMF04	0	0	1
	AMF02	1	1	1		AMF05	0	0	1
	AMF03	1	1	1		AMF06	1	1	1
	AMF04	1	1	1		AMF07	1	1	1
	AMF05	1	1	1		AMF08	1	1	1
	AMF06	1	1	1	OPC19	AMF01	0	1	1
	AMF07	1	1	0		AMF02	1	1	0
	AMF08	1	1	1		AMF03	1	1	1
	AMF09	1	1	1		AMF04	1	1	1
OPB04	AMF01	1	1	1		AMF05	1	1	1
	AMF02	0	1	0		AMF06	1	1	1
	AMF03	1	1	1		AMF07	1	1	1
	AMF04	1	1	1		AMF08	1	1	1

1: Present. 0: Absent.

Table 5: DNA fragments amplified from the DNA extracts of three different strains of *M. tuberculosis* (A, B and C) using sixteen RAPD-PCR primers.

RAPD-PCR primers	Total amplified fragments	*M. tuberculosis* strains		
		M0-041 (A)	H37Ra (B)	M03-035 (C)
OPA03	6	5	3	5
OPA06	4	3	2	3
OPA09	6	5	4	4
OPA10	6	4	3	4
OPA11	6	6	5	6
OPA16	3	3	3	3
OPA17	11	11	5	4
OPA19	9	8	6	7
OPB04	6	6	4	5
OPB07	5	5	5	4
OPB08	6	5	5	3
OPB15	11	8	8	10
OPB20	8	7	5	8
OPC05	7	7	5	6
OPC07	8	8	7	8
OPC19	8	7	8	7
Total	110	96	78	87

Table 6: DNA unique (markers) fragments amplified from the DNA extracts of three different strains of *M. tuberculosis* (A, B and C) using sixteen RAPD-PCR primers.

RAPD-PCR primers	Present DNA unique markers			Absent DNA unique markers		
	M. tuberculosis strains			*M. tuberculosis* strains		
	M0-041 (A)	H37Ra (B)	M03-035 (C)	M0-041 (A)	H37Ra (B)	M03-035 (C)
OPA03	1	-	1	-	1	-
OPA06	1	-	1	-	-	-
OPA09	1	1	-	-	1	-
OPA10	1	1	1	-	1	-
OPA11	1	-	-	-	1	2
OPA16	-	-	1	-	-	-
OPA17	1	-	-	-	3	2
OPA19	-	-	-	-	-	1
OPB04	-	1	1	-	1	-
OPB07	-	-	-	-	1	-
OPB08	1	-	-	-	1	-
OPB15	-	-	-	-	1	1
OPB20	4	-	-	-	1	-
OPC05	-	1	-	-	1	2
OPC07	-	-	2	-	1	-
OPC19	-	-	-	1	-	1
Subtotal	11	4	7	1	14	9
	22			24		
Total	46					

-: No unique markers were recorded.

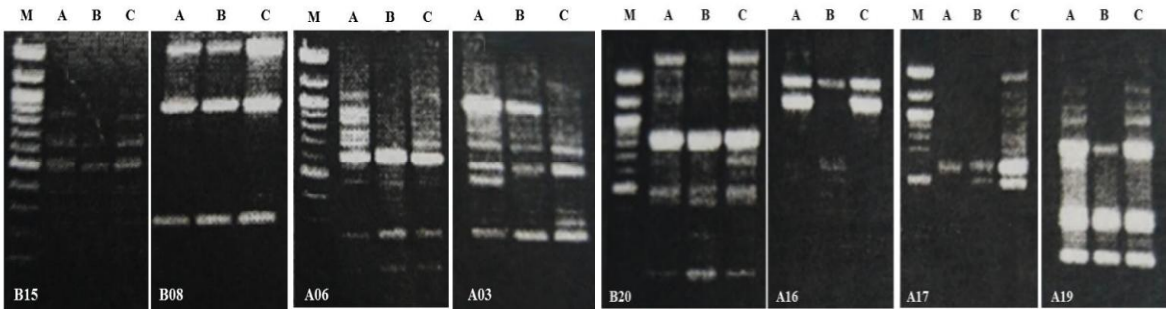


Figure 6: electrophoresis of agarose gel (1.2%) in tae buffer stained with ethidium bromide shows the DNA polymorphisms produced via RAPD-PCR using DNA templates of three different of m. tuberculosis strains (M0-041 (A); H37Ra (B) and M03-035 (C) and eight RAPD-PCR primers of operon a and b groups. m: DNA marker.

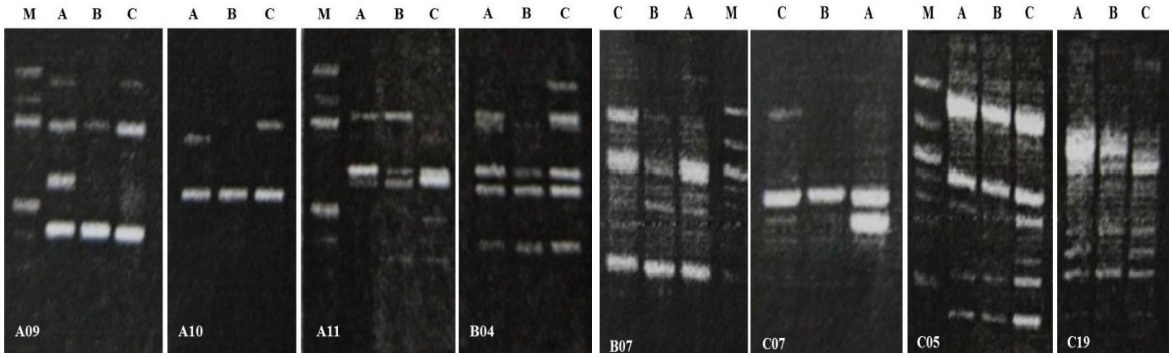


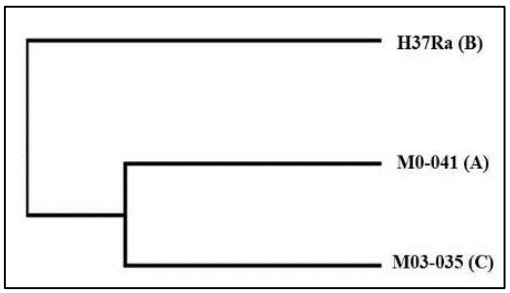
Figure 7: Electrophoresis of agarose gel (1.2%) in TAE buffer stained with ethidium bromide shows the DNA polymorphisms produced *via* RAPD-PCR using DNA templates of three different of *M. tuberculosis* strains (M0-041 (A); H37Ra (B) and M03-035 (C) and eight RAPD-PCR primers of Operon A, B and C groups. M: DNA marker.

Statistical analysis of RAPD-PCR polymerphisms revealed a degree of similarities with ratio from 80 to 85.4% between the TB strains (Table 7). The phylo-genetic tree in Figure-8 showed that strains A and C obtained from patient's represent-ted a cluster with similarity of 85.4%. While, the TB standard strain (B) created a separated cluster with 80.0-83.3% identity.

Table 7: Similarity (%) between three different strains of *M. tuberculosis* (A, B and C) based on DNA polymerphisms amplified from their DNA extracts using sixteen RAPD-PCR primers.

M. tuberculosis strains	M0-041 (A)	H37Ra (B)	M03-035 (C)
M0-041 (A)	100		
H37Ra (B)	83.0	100	
M03-035 (C)	85.4	80.0	100

Figure 8: Phylogenetic tree of three different strains of *M.*



tuberculosis based on DNA RAPD-PCR analysis of DNA polymorphisms amplified from their DNA extracts using sixteen RAPD-PCR primers.

The use of RAPDs for typing of *Mycobacterium* has documented by several investigators (Abed *et al*., 1995 a and b; Rodriguez *et al*., 1995; Harn *et al*. 1997 and Richner *et al*. 1997). Collyns *et al*., (2002**)** supported the present study, particularly; they reported that molecular finger-printing of *M. tuberculosis* could be useful for understating the epidemiology of tuberculosis.

This study recommended the use of RAPD-PCR as a molecular tool for characterization of bacteria for their rapid and simply procedure as well as low material demands. Most importantly, nonprevious nucleotide sequence information is needed for the construction of primers.

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