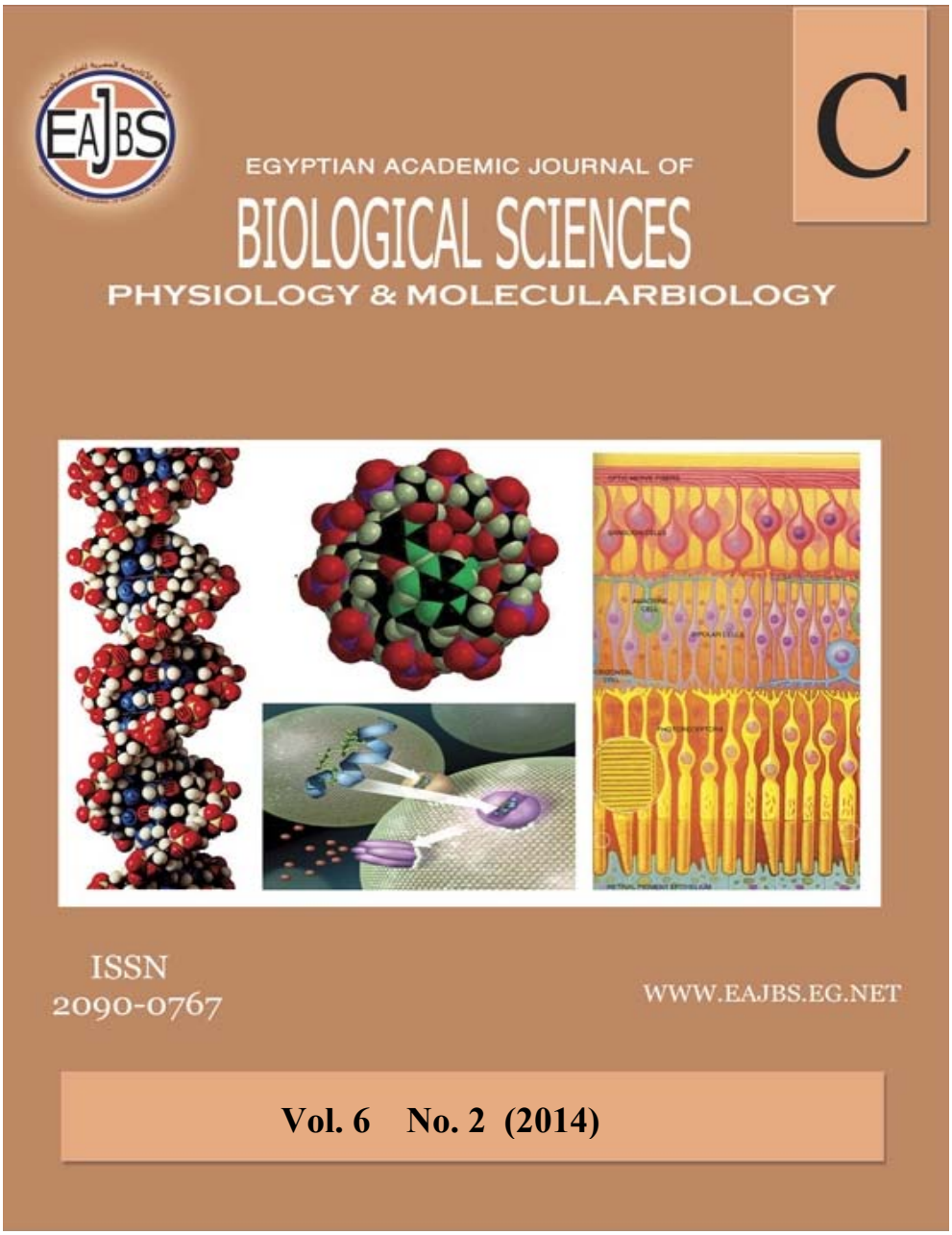

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Molecular Characterization of Mycobacterium tuberculosis Using Polymerase Chain Reaction

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ABSTRACT

Objective: The purpose of this study was to isolate and characterize Mycobacterium tuberculosis in Khartoum state using Polymerase Chain Reaction technique.

Methodology:71 specimens of sputum were collected from different hospital in Khartoum state were positive with Ziehl Neelsen stain.specimens were decontaminated and inoculated on Lowenstein Jensen media according to modified Pettrof's method, biochemical tests, DNA was extracted from each specimen, and then the Polymerase Chain Reaction technique was adopted to detect Insertion Sequence IS6110 gene of M. tuberculosisin these specimens.

Results: Out of 71 samples, 57 (80.2%) of the isolates were Mycobacterium tuberculosis complex organisms, biochemical tests showed 68 (95.8%) were sensitive for Para-nitrobenzoic acid; 56 (78.9%) were resistant to Thiophene–2Carboxylic Acid Hydrazide (TCH); 68 (95.8%) were positive for nitrate reduction and 65 (91.6%) were catalase negative at 68°C. while 5(7.1%) revealed no growth. Sixty six Mycobacterium tuberculosis complex isolates were subjected to PCR. Fifty two (78.8%) showed that the positive results while, Fourteen (21.2%) of specimenc were negative by PCR.

Conclusion: This results revealed clearly the importance of conventional methods including Z.N stain and culture techniques in the diagnosis of TB especially if there is other invaders like Mycobacteria other than tuberculosis are suspected.

INTRODUCTION

Tuberculosis is one of the leading infectious diseases in the world and is responsible for more than 2 million deaths and 8 million new cases annually (Graham, *et al*. 2004). Because of the slow growth rate of the causative agent *Mycobacterium tuberculosis*, isolation, identification, and drug susceptibility testing of this organism and other clinically important mycobacteria can take several weeks or longer.

During the past several years, many molecular methods have been developed for direct detection, species identification, and drug susceptibility testing of mycobacteria. These methods can potentially reduce the diagnostic time from weeks to days (Soini and Musser, 2001).Rapid diagnosis and treatment are important for preventing transmission of *Mycobacterium tuberculosis*. However, the diagnosis of tuberculosis continues to pose serious problems, mainly because of difficulties in differentiating between patients with active tuberculosis and those with healed lesions, normal *Mycobacterium bovis* BCG (Bacillus Calmette Guerin) vaccinated individuals, and unvaccinated Manteux positives. Physicians still rely on conventional methods such as Ziehl-Neelsen (ZN) staining, fluorochrome staining, sputum culture, gastric lavage, and other non-traditional methods (Marais, *et al.* 2006). Although the tuberculin test has aided in the diagnosis of tuberculosis for more than 85 years, its interpretation is difficult because sensitization with non-tuberculous mycobacteria leads to false-positive tests (Graham, *et al*. 2004). The detection of mycobacterial DNA in clinical samples by polymerase chain reaction (PCR) is a promising approach for the rapid diagnosis of tuberculous infection. However, the PCR results must be corrected for the presence of inhibitors as well as for DNA contamination (Garg, *et al.*2003).

MATERIALS AND METHODS

This comparative study was conducted in the Department of Tuberculosis, National Health Laboratory, Khartoum, Sudan, from May 2009 to December 2009, on sputum specimens of 71patients suspected of pulmonary tuberculosis. samples were collected according to WHO criteria (1998) in sterile, plastic, wide-mounted and leak-proof containers, wrapped with sufficient absorbent material to soak up all the specimen should the container break and within a robust outer plastic box of cardboard. Sputum samples were not saliva but coughed up from the lungs after three times mouth wash. Samples were processed in the same day when received.

Ziehl Neelsen stain

Purulent particle of sputum specimen were selected. A loopful of sputum was spread on clean glass slide. Smear was left to dry completely and fixed by passing through the flame carefully. The proper temperature for flame fixation was checked by touching the slide to the back of hand immediately after removing it from the flame. Smear was covered with carbol fuchsin and heat gently until vapour raised, the heating stain allowed for 5 minutes and washed off with clean water. Decolourization was done by 3% acid alcohol for few seconds. Washed and then malachite green was added for 2 minutes.Washed, dried and examined under microscope.

Culture Method

Two tubes of Lowenstein Jensen medium were inoculated with 20 µl of the sediment that was obtained from the digestion and decontamination of the sputum sample. One of the two tubes contains glycerol while the other tube contains pyruvic acid to isolate the M. bovis species if present. All cultures were incubated at 37°C for 8 weeks before being discarded. Growth was monitored daily during the first week to observe the presence of rapid growers which if present will show growth within 7 days, and then the growth was observed weekly up to the 8th week.

Identification of Isolate:

A suspension of culture under test was inoculated onto LJ slope, the organism is either slow grower, if it produce visible colonies on subculture usually after 2-6weeks incubation or rapid grower, if it produce visible

colonies on subculture with 5 days incubation (usually after 2-3 days).

***Catalase Test*:**

In screw capped tube. Several loopful of test culture were suspended in the buffer solution. Tubes containing the emulsified cultures were placed in a previously heated water bath at 68°C for 20 minutes, time and temperature was critical. Tubes were removed from heat and allowed to cool to room temperature. 0.5 ml of the freshly prepared tween – pyroxide mixture was added to each and caps were placed loosely. Formation of bubbles was observed appearing on the surface of the liquid, the tubes should not be shake because tween 80 also may form bubbles when shaken resulting in false positive results. Negative tubes were holded for 20 minutes before discarding.

***Nitrate Reduction Test*:**

A heavy loopful of a recently grown culture on L.J. media was transferred to tube containing solution of sodium nitrate. The suspension was incubated for 3 hours at 37°C. Small quantity of LAMP reagent was added.

Sensitivity to Para–Nitrobenzoic Acid (PNB) 500 mg/L: Loopful of homogenized culture suspension was inoculated onto slope of medium containing PNB 500 mg / L and observed for growth during an incubation period of 4 weeks at 37°C.

***Sensitivity to Thiophene- 2 Carboxylic Acid Hydrozide (TCH) 5mg/L*:**

Loopful of homogenized culture suspension was inoculated onto slope of medium containing TCH 5 mg / L and observe for growth during an incubation period of 4 weeks at 37°C.

DNA Extraction from Cultures:

Different protocols for DNA extraction were used but DNA was extracted by 10% Chelex by adding 150 µl of 10% Chelex into eppendorf tube and boiled in water bath for 10 minutes. Then, 10 µl of the organism suspension was added and mixed well. The mixture was boiled again for 10 minutes then centrifuged at 12000 rpm for 5 minutes and this step was repeated after transferring the supernatant to new eppendorf tube, then was kept in -20°C till use. Amplification of insertion sequence IS6110 (123 bp) (Eisenach *et al*., 1990) was performed with a set of primers having the following sequence: Forward:
(CCTGCGAGCGTAGGCGTCGG)
Reverse:
(CTCGTCCAGCGCCGCTTCGG). A master mix reagent was prepared for 15 reactions according to (Eisenach, *et al.*1990), which contained:82.5 µl PCR buffer (PROMEGA), 25 mM MgCl2 (PROMEGA), 15 µl from each dNTP (100mM) (PROMEGA), 1 µl of each primer (Inqaba Biotec), 3 µl Taq polymerase (5U/µl) (PROMEGA), 480 µl distilled water and 3µl template DNA. *M. tuberculosis* strain H37R was used as positive control. The negative control contained reaction mixture without template DNA.

PCR amplification

The amplification was done using a thermal cycler from TECHNE (UK). A PCR program was conducted with an initial 5 minutes denaturation step at 94°C for one cycle followed by a repeating cycle of denaturation (2 minutes at 94°C), annealing (2 minutes at 68°C) and extension (2 minutes at 72°C) for 25 cycles, followed by a 5 minutes final extension step at 72°C.The amplified products were resolvedby electrophoresis on the 2% agarose gel andstained with ethidium bromide andvisualized on a UV. Transilluminator.

RESULTS

From the collected 71 sputum samples, 57 showed MTC-like colonies, 9 were considered rapidly growing mycobacteria and 5 samples revealed no growth. The MTC-like colonies were confirmed by some of their phenotypic and genotypic characteristics.The growth

rate of the isolates ranged between 3 days and 5 weeks. Most of the isolates showed visible growth after 2 weeks. Nine out of 71 isolates were identified as rapid growers of mycobacteria, while the growth rate of 57 isolates ranged between 2 to 5 weeks and they were identified as slow growers and considered belonging to MTC species.

Biochemical tests: Out of 71 samples, 57 isolates of MTC organisms (slow growers), were sensitive for para-nitrobenzoic acid (growth inhibited by PNB); resistant for Thiophene-2-Carboxylic Acid Hydrozide TCH; positive for nitrate reduction (reduce nitrate to nitrite) and negative for catalase at 68°C.

Polymerase chain reaction: The Sixty six Mycobacterium tuberculosis complex isolates which showed rapid or slow grower were subjected to PCR. Fifty two isolates showed a band typical in size (123 bp) to the target gene (IS 6110) as indicated by the standard DNA marker. 14 isolates were negative (Table 1 and Fig.1).

Table 1: Shows correlation between PCR and Culture on LJ media

		Results		Total
		Negative	Positive	
Diagnostic tool	Culture	9(13%)	57(86.3%)	66(100%)
	PCR	14(21.2%)	52(78.8%)	66(100%)

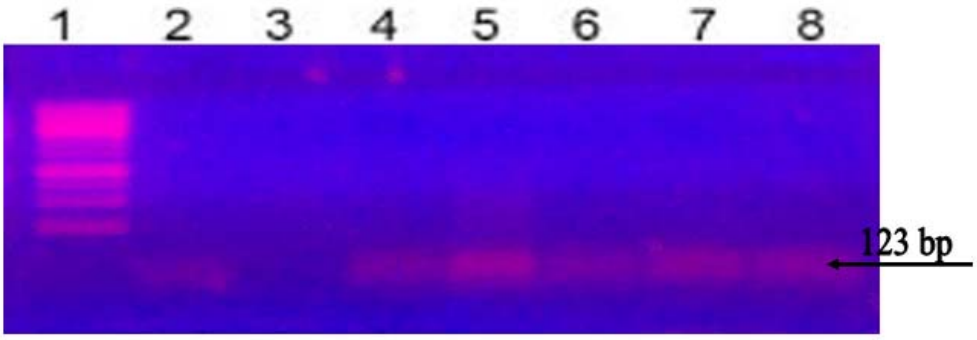

Fig. 1: The amplicon of MTC after run of PCR on 1 % agarose gel as follow: Lane 1 = marker; 3 = control negative; 2, 4, 5, 6, 7 and 8 = positive for MTC (123 bp band).

DISCUSSION

Tuberculosis is one of the leading infectious diseases in the world and is responsible for more than 2 million deaths and 8 million new cases annually (Soini and Musser, 2001). In much of the world, tuberculosis is the leading cause of death from any one infectious agent, directly responsible for an estimated 7% of all deaths and 26% of all preventable deaths worldwide (McPherson and pincus, 2006).The conventional methods used for diagnosing TB infection are unable to distinguish between M. tuberculosis strains causing reactivation or reinfection. Phenotypic markers currently used to distinguish between bacterial strains have limited discriminatory ability for M. tuberculosis; results may not correlate with genotypic methods and are not widely used (Batool, 2006).From seventy one positive AFB sputum samples, 57 (80.2%) isolates of M .tuberculosis were identified as slow growing MTB and 9 (12.7%) samples were considered as rapidly growing mycobacteria, 5 (7.1%) samples gave no growth, this may due to contamination or improper technique. Biochemical tests for identification of M. tuberculosis complex showed that 56 (78.9%) of isolates were resistant for

TCH; 68 (95.8%) were sensitive to PNB; 68 (95.8%) were positive for nitrate reduction test (reduce nitrate to nitrite) and 65 (91.5%) were negative for catalase test. Biochemical tests results were in range according to WHO criteria (1998).PCR assay characterized 66 of the examined isolates as M. tuberculosis complex species. The sensitivity of the PCR reached 78.8% compared with the LJ medium results. 52 (78.8%) gave positive results (typical band at 123 bp) and 14 (21.2%) were negative (never gave a band). These findings agree with the 75% sensitivity that was found by Cormican et al., (1995), the 87.5% which was found by Tonjum et al., (1996) and the 83.6% which was found by Garg et al., (2003). PCR was negative for an additional 5 (7.1%) isolates which were positive by ZN smear and culture. No positive results for the M. tuberculosis complex was obtained by the PCR for the isolates which were identified as MOTT or other bacterial species grown on LJ media. Acid-fast smears have high specificity but some other organisms may also stain acid-fast, including Nocardia, Rhodococcus and Legionella micdadei.

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ARABIC SUMMARY

التمييز الجزيئي للمتفطرة السُّلّيِة بإستخدام تفاعل البلمرة التسلسلي

بشير سراج بشير [١] و مجاهد محمد الحسن[٢]
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الهدف:هدفت هذه الدراسة للتعرف والتمييز للمتفطرة السُّليِّة لدى مرضى الدرن الرئوي في ولاية الخرطوم باستخدام تفاعل البلمرة التسلسلي.
طرق القياس: تم جمع 71 عينة من البلغم من مستشفيات ولاية الخرطوم كانت إيجابية بصبغه زيل نلسون.تمتعملية إزالة التلوث من جميع العينات ومن ثمتمت زريعها بوسط لونستنجون سنبناء على طريقة بترو فالمعدلة. تم إجراء الاختبارات الكيميائية كما تم استخلاص الحمض النووي للجرثومة ومنثمتم إجراء تقنية تفاعل البلمرة المتسلسلل لكشف عن جين المتفطرة السلية.
النتائج: من جملة واحد وسبعين عينة، ٥٧ (٨٠.٢%) من العينات المعزولة من عضيات الباكتيريا المتفطرة الدرنية. الاختبارات الحيوكيميائية أظهرت ٦٦ (٩٣%) كانت حساسة لإختبار حمض البارانايتروبنزويك (الحمض يمنع نمو الباكتيريا)، ٥٦ (٧٨.٩%) كانت مضادة لإختبار الثيوفين - ٢ - كاربوكسيلك اسيد هيدرازايد، ٥٧ (٨٠.٢%) كانت موجبة لإختبار إختزال النترات و ٦٥ (٩١.٦%) من العينات المعزولة كانت سالبة لإختبار الكاتاليز بينما ٥ (٧.١%) لم تظهر نموا. ستة وستين عزلة من عضيات الباكتيريا المتفطرة الدرنية من المعمل المرجعي للدرن تم إختبارها بواسطة تفاعل البلمرة التسلسلي. اثنان وخمسون (٧٨.٨%) عزلة كانت ايجابية بينما أظهرت ١٤ (٢١.٢%) عينة نتائج سالبة.
الاستنتاج: هذه الدراسة كشفت بوضوح أهمية الطرق التقليدية التي تشمل تقنيات صبغة زيهل نيلسن والتزريع في تشخيص الدرن خصوصا إذا وجدغزاة آخرين مثل المتفطرات الأحرى غير المتفطرة الدرنية اشتبه فيها.