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Polymerase Chain Reaction as a Rapid Tool for the Diagnosis of Pulmonary Tuberculosis

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ABSTRACT

Objective: This study aimed to use the polymerase chain reaction (PCR) as a rapid tool for the diagnosis of pulmonary tuberculosis from sputum samples. Clinically suspected tuberculosis patient in Khartoum state were targeted.

Materials and Methods: Sputum specimens were collected from patients attending Abu Anga Hospital, Alsha'ab Teaching Hospital and Tuberculosis Reference Laboratory. Patients were consented and informed. Sputum samples were stained with ZN stain, then decontaminated and cultured on LJ medium. Part of the sputum was used for DNA extraction by isopropanol method for PCR.

Results: 37(21.6%) smears from the collected 171 sputum samples showed positive ZN smears while 134 (78.4%) showed negative result. On LJ medium, 23.4% showed MTC-like colonies, 5.8% were considered rapidly growing *Mycobacteria* and 70.8% samples revealed contamination or no growth. The MTC-like colonies were confirmed by conventional methods.

When PCR was performed, 142 (83%) samples showed a band typical in size (123 bp) to the target gene of *Mycobacterium tuberculosis* complex (*IS 6110*) as indicated by the standard DNA marker. 29(17%) samples were negative.

Conclusion: These results revealed clearly the importance, feasibility and sensitivity of PCR as a rapid diagnostic tool to detect *M. tuberculosis* directly from sputum samples.

Key words: tuberculosis, IS 6110 gene, Sudan, TB diagnosis

INTRODUCTION

Tuberculosis remains a major global health problem. The actual global prevalence of *Mycobacterium tuberculosis* infection is 32%, corresponding to approximately 1.9 billion people. According to the world health organization there were 8.8 million estimated new cases (case rate 140/100,000) of pulmonary tuberculosis. An estimated 1.9 million people died of tuberculosis, including patients co-infected with human immunodeficiency virus (HIV) (Murray *et al*., 2007).

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 (Garg *et al*., 2003). Physicians still rely on conventional methods such as Ziehl-Neelsen (ZN) staining, fluorochrome staining, sputum culture, gastric lavage, and other non-traditional methods. Tuberculosis Skin Test (TST) has been in use for the diagnosis of tuberculosis infection since 1910. TST or intradermal Manteux is the oldest diagnostic test and despite the

diagnostic limitations it is included in the WHO latest recommendations for TB control. TST is based on a protein–purified derivate (PPD), resulting from a culture filtrate of tubercle bacilli containing over 200 antigens common both in bacilli Chalmette-Guerin vaccine (BCG) and in most non tuberculosis bacteria. Consequently, the TST specificity is low and also the ability to distinguish latent TB infection is limited (Tsara *et al.,* 2009). Although the conventional technique of direct smear examination with Ziehl-Neelsen staining (ZN) is cheap and easy to perform; its low sensitivity is a major drawback. Depending on the number of specimens examined, ZN detects 30 to 60% of the culture-positive "TB suspects. Furthermore, it requires sputum samples collected on consecutive days, making the procedure slow and making patient compliance with the diagnostic process difficult (Ndugga *et al.,* 2004).

The microbiological culture, generally used in suspected pulmonary cases and sputum smear microscopy negative cases, has the advantage of allowing the detection and isolation of the Mycobacteria, the identification of the species and/or of the isolated complex, and the determination of the sensitivity of the microorganism to chemotherapeutic agents for TB. The principal culture media used are Lowenstein-Jensen (egg-based solid medium) and Middlebrook (solid or liquid, in agar medium). Despite its importance, the culture of *Mycobacterium tuberculosis* is time-consuming, due to the slow growth of the bacillus (15-20 h), and the test does not always present 100% positivity (Teixeira *et al.,* 2007).

Several immune based tests have also been developed aiming to improve diagnostic procedures of TB infection. Serologic tests are commercially available and they are based on the detection of the antibody immune response to *Mycobacterium tuberculosis*. The antibody detection kits differ in their features, depending on the kind and the site of the target antigen and also on the incubation techniques. Systematic reviews, regarding antibody detection tests, have showed that they vary widely in performance and sensitivity (Tsara *et al.,* 2009).

Molecular technique such as the polymerase chain reaction (PCR) permits the direct detection and identification of infectious agents in clinical specimens, saving days to weeks in diagnostic time. Its application to infectious disease caused by fastidious or slow growing microorganism, such as *M. tuberculosis*, has the potential to provide a truly rapid laboratory diagnosis with the attended improvement in patient management and reduction of medical costs (Amato and Miller. 2008).

Early diagnosis of the causative agent followed by adequate treatment is essential to prevent both morbidity and mortality (Ndugga *et al.,* 2007).

The TB diagnostic techniques currently used are slow and have sensitivities and specificities that need to be improved. Although the presumptive diagnosis of TB may be obtained through clinical history and radiological findings, the final diagnosis still depends on smear and culture.

The most promising technique for a fast diagnosis is based on the polymerase chain reaction (PCR), which is theoretically capable of detecting one copy of DNA of any cell. Besides the high sensitivity and specificity, this technique can produce results in few hours, and is being used in the diagnosis of a number of infectious diseases.

The study aimed to use polymerase chain reaction (PCR), as rapid tool for the diagnosis of pulmonary tuberculosis from sputum samples.

MATERIALS AND METHODS

Study Population and sampling

One hundred and ninety four suspected patients attending Abu Anga Hospital, DOTS (Directly Observed Treatment, Short course) reference Tuberculosis Laboratory and Elasha'ab Teaching Hospital were recruited to this study.

Data were collected using a standardized questionnaire eliciting information on symptoms of tuberculosis, risk factors, social history, history of previous tuberculosis, tuberculosis treatment, and tuberculin skin testing. Sputum samples were collected in clean, wide mouthed, and leak proof specimen containers.

A direct smear was made from each sputum specimen, stained by the ZN method, and read at the center. After that, the specimen was sent as soon as possible (i.e., < 24 hours) to reference Tuberculosis Laboratory for culture and DNA extraction.

Sputum samples were decontaminated before culture and DNA extraction.

Sputum samples were then cultured on LJ medium. 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 was made by growth rate and biochemical reactions.

Molecular Identification (PCR)

Polymerase chain reaction was used to diagnose pulmonary tuberculosis from direct sputum samples.

DNA was extracted by Isopropanol extraction method by mixing 100 ul of decontaminated sample in eppendorf tube (1.5 ml) with 400 ul of lysis buffer and 300 ul of Isopropanol, centrifuged at 12000 rpm for 10 minutes, the supernatant was decanted by gently inverting the tube, then the pellet was washed by 75% Ethanol, and re centrifuged at 12000 rpm for 5 minutes, the previous step was repeated three time, the final pellet was re-suspended in 50 ul Tri EDTA 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 100 reactions according to (Eisenach et al., 1990), 5µl template DNA from each sample was added to the master mix. The negative control contained reaction mixture without template DNA.

A PCR program was conducted with an initial 5 minutes denaturation step at 94°C for one cycle followed by a repeating cycles 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 of final extension at 72°C. Then, the PCR product was visualized by UV transilluminator on 1% agarose gel.

RESULTS

Among the study population (171 patients), 69.6% were found to be males, and 30.4% were females.

All the ages were found to be affected with tuberculosis. However, the highest frequency was found among age group 21-50 years (68.5%), followed by age >50 (18.2%) while the lowest frequency was among age group <20 (13.3%).

Smear positive samples were 37(21.6%) while 134 (78.4) showed negative result.

When the 171 sputum samples were cultured on LJ medium, 23.4% showed MTC-like colonies, 5.8% were considered rapidly growing mycobacteria and 70.8% samples revealed contamination or no growth. The MTC-like colonies were confirmed by conventional methods.

The growth rate of the isolates ranged between 3 days and 5 weeks. Most of the isolates showed visible growth after 2 weeks. Ten out of 171 isolates were identified as rapid growers mycobacteria, while the growth rate of 40 isolates ranged between 2 to 5 weeks and they were identified as slow growers and considered belonging to MTC species.

Indirect smears from suspected MTC, rapid growers of mycobacteria were all found positive for AFB.

Cultural properties of all isolates of *Mycobacterium tuberculosis* complex on Lowenstein Jensen medium at 37°C were almost the same and all colonies were found dry, rough and buff colored.

Out of the 171 samples, 40 isolates of MTC organisms (slow growers), were positive for nitrate reduction and negative for catalase at 68°C.

All sputum sample were extracted by Isopropanol method and were subjected to PCR, 142 samples showed a band typical in size (123 bp) to the target gene (*IS 6110*) as indicated by the standard DNA marker. 29 samples were negative, as shown in Fig.1 below.

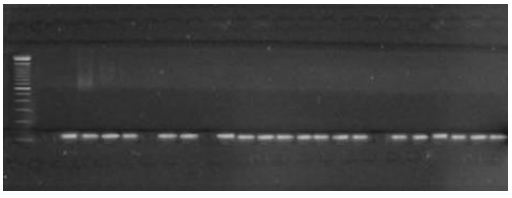

Fig. 1: The amplicon of MTC after PCR run on 1 % agarose gel: Lane 1 = marker; 2 = negative control 2, 3, 4 ,5 ,6 ,8, 9, 11, 12, 13, 14, 15, 16, 17, 18,20,21,22,23,24 and 25 are positive for MTC (123 bp band) 7,10 and 19 are negative for MTC.

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 among all infectious agents, directly responsible for an estimated 7% of all deaths and 26% of all preventable deaths worldwide (McPherson and Pincus, 2006).

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).

The aim of the present study was to use the polymerase chain reaction (PCR), as a rapid tool for the diagnosis of pulmonary tuberculosis from direct sputum.

Preparing DNA by using Isopropanol extraction method simplified the procedure (Not contain biohazards and take 30 minutes) so that it can be used in routine clinical practice.

Among the total of 171 specimens studied, 37(21.6%), were smear positive, 40 (23.4%) were culture positive. Smear microscopy was positive in only 12 (30%) of the culture positive samples. This may be due to the low sensitivity of ZN stain to detect AFB which needs more than 500 bacilli/ml (Iqbal, 2003). In

comparison, PCR was positive in 95% of the culture positive. This determines the high sensitivity of PCR to detect culture positive. This finding is similar to (Kavita Modi, 2006) who found the sensitivity of smear and culture as 51% and 68% respectively. Smear microscopy was positive in only 67% of the culture positive samples while PCR was positive in 98%.

Twenty (20) samples which were ZN positive, PCR positive but cultures negative were from patients with clinical manifestations of TB. This could be due to the presence of non-viable mycobacteria in the samples as some of the subjects were receiving anti tubercular treatment. This finding is in accordance with a report from (Luciano dos, 2003) who found that ten patients with clinical manifestations of TB were culture-negative but PCR-positive.

84 (67.4%) samples which were smear and culture negative obtained from patients clinically suspected of *M. tuberculosis* were PCR positive, suggesting that PCR assay is probably more sensitive than the culture by detecting non-viable and/or fewer viable organisms. Similar results were obtained by Aroma Oberoi, (2007) who found that PCR showed the highest sensitivity (73.9%) as compared to other tests.

Two (8.3%) culture-positive samples were PCR negative. This may indicate the presence of PCR inhibitors in these samples. This finding is similar to (Maher, 1996) who found that two culture-positive samples were PCR negative.

In conclusion, isopropanol extraction method is effective, safe and rapid procedure for DNA extraction from sputum sample.In addition, PCR is highly sensitive, effective and much more rapid than conventional methods in diagnosis of pulmonary tuberculosis.

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ARABIC SUMMARY

استخدام تفاعل البلمرة التسلسلي، كاداة سريعة لتشخيص السل الرئوي

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هدفت الدراسة إلى استخدام تفاعل البلمرة التسلسلي، كاداة سريعة لتشخيص السل الرئوي من عينات التفاف عند المرضى الذين يعانون من اعراض السل في ولاية الخرطوم.

تم جمع عينات التفاف من مستشفي ابوعنجة، ومستشفي الشعب التعليمي، و المعمل القومي الصحي. وقد تم تنوير المرضى بهذه الدراسة واخذ موافقتهم. 37 (21.6%) مسحة من مجمل 171 عينة تفاف اظهرت نتيجة موجبة، بينما 134(78.4%) اظهرت نتيجة سلبية لصبغة العصويات المقاومة للاحماض. كل عينات التفاف تم تزريعها في وسط ليونيستين جنسن، 23.4% اظهرت مستعمرات تشبه عضيات الباكتيريا المتفطرة الدرنية، 5.8% اعتبرت متفطرة سريعة النمو و 70.8% من العينات اظهرت تلوثا اولم تنمو. المستعمرات التي تشبه المتفطرة الدرنية تم تاكيدها بواسطة الطرق التقليدية.

وتم استخراج الحمض النووي الريبي منقوص الأكسجين من جميع عينات التفاف بطريقة الأيسوبروبانول وتم إختبارها بواسطة تفاعل البلمرة التسلسلي، 142 (83%) عينة أظهرت حزمة مطابقة في القياس للجين المستهدف كما هو مشار إليه بواسطة المؤشر القياسي للحامض النووي الرايبوزي منزوع الأوكسجين، بينما اظهرت 29 (17%) عينة نتائج سلبية.

وأظهرت النتائج بوضوح اهمية، وحساسية، وجدوى تفاعل البلمرة التسلسلي كأداة سريعة للتشخيص والكشف عن المتفطرة السلية من عينات التفاف.