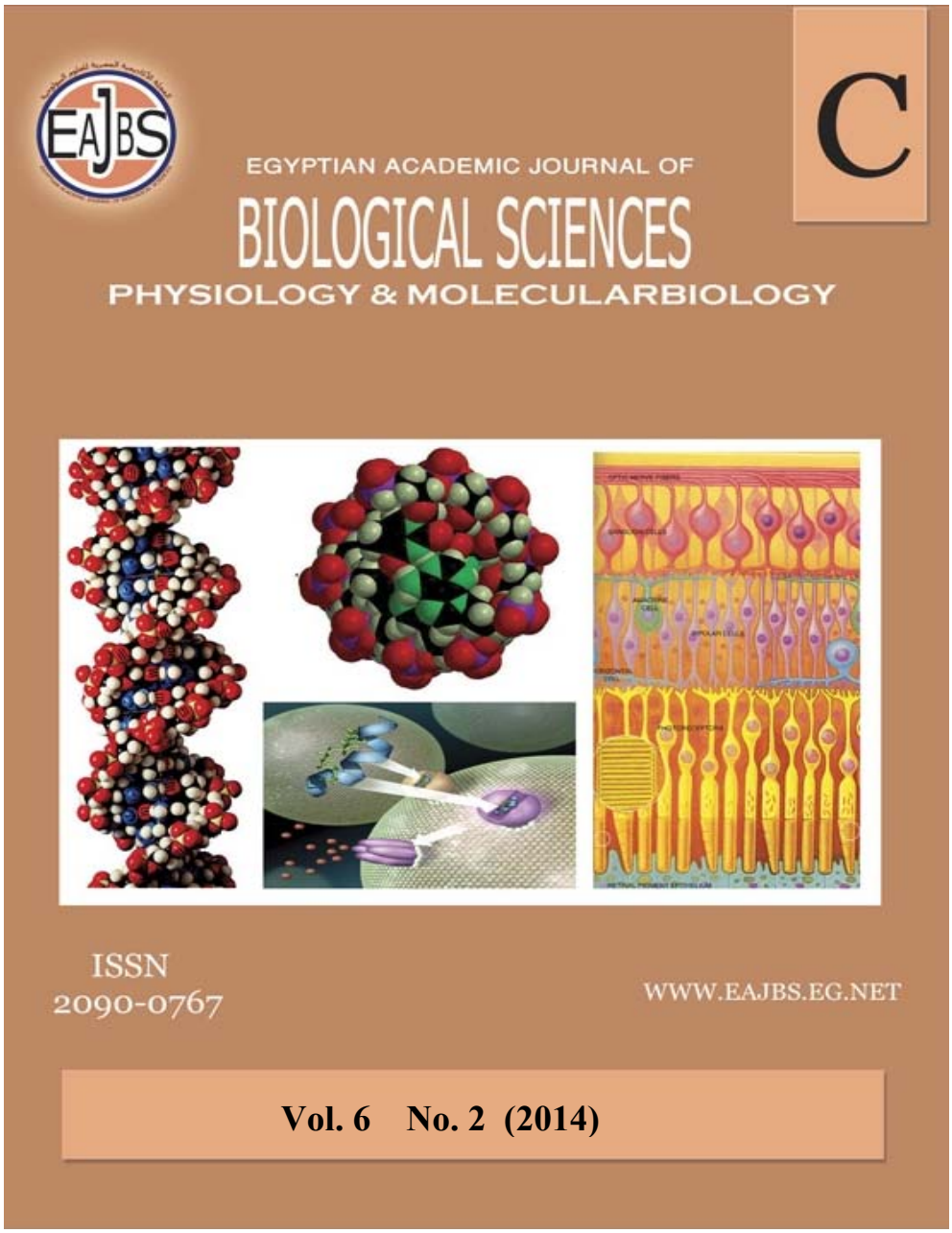

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Improved Detection of *Mycobacterium tuberculosis* in Sudanese Children by use of Insertion Sequence IS6110

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ABSTRACT

This study was carried out in Khartoum state during the period from January 2010 to December 2013 to detect *Mycobacterium tuberculosis* in children with symptoms of tuberculosis infection using different conventional and advanced diagnostic techniques. One hundred ninety seven specimens of gastric lavage and sputum were collected from different hospital in Khartoum state**.**specimens were decontaminated and inoculated on Lowenstein Jensen media according to modified Pettrof's method, two smears were prepared and stained by Ziehl Neelsen stain bacterial DNA was extracted from each specimen by using phenol chloroform method, and then the Polymerase Chain Reaction technique was adopted to detect Insertion Sequence IS6110 gene of *M. tuberculosis*in in these specimens.

This study showed that the positive results for ZN, Culture and PCR were 16 (8.1%), 32 (16.2%) and 35(17.8%) respectively. The study concluded that the PCR technique is a most sensitive and specific technique for a fast identification of *M. tuberculosis* in gastric lavage and sputum from children who are unable to expectorate good quality sputum sample or diagnosed as negative using conventional diagnostic methods.

INTRODUCTION

Tuberculosis (TB) is a chronic contagious disease which has a major impact on global public health problem. The disease is caused by *Mycobacterium tuberculosis* (MTB). Tuberculosis kills ~2 million people each year, and in 1989, the World Health Organization estimated that ~300,000 children < 15 years of age die of tuberculosis per year worldwide (WHO–WHO/TUB, 2003). Tuberculosis (TB) remains a leading cause of mortality in children worldwide.

The diagnosis of tuberculosis in children is traditionally based on exposure history, symptoms, the tuberculin skin test, chest radiography, and mycobacterial staining and culture. However, these investigations lack in sensitivity and specificity and diagnosing childhood tuberculosis remains a major global challenge.

The diagnosis of pulmonary tuberculosis presents challenges in children, because symptoms are nonspecific, sputa are not accessible, and *Mycobacterium tuberculosis* cultures and smears often are negative. The diagnosis of childhood tuberculosis is often problematic and depends on a combination of epidemiological factors, clinical and radiological features, and/or bacteriologic confirmation. Bacteriologic confirmation is not routinely attempted in children with possible TB and specimens for mycobacterial culture are collected only in children admitted to hospital (Graham *et al*., 2004).

Few studies have reported as high as 33% bacteriological positivity of childhood tuberculosis even in primary disease such as hilar lymphadenopathy. This contradicts the concept of primary tuberculosis, which we understand till date as being difficult to diagnose by demonstration of AFB due to its paucibacillary nature, and the fact that Ziehl-Neelson stain can reveal AFB only if the sample contains >10,000 bacilli per mL (Working Group on Tuberculosis, 2010).

Although some previous studies demonstrated the role of molecular diagnosis of tuberculosis, there has been no previous study reported showing the role of molecular diagnosis of childhood tuberculosis by detection of specific gene in gastric aspirate in Sudan.

This study helps in detection of childhood tuberculosis cases by using Polymerase Chain Reaction (PCR), the vast majority of them have smear-negative disease by using conventional techniques used in diagnosis of childhood tuberculosis.

MATERIALS AND METHODS

This comparative study was conducted in the Department of Tuberculosis, National Health Laboratory, Khartoum, Sudan, from May 2011 to December 2011, on gastric aspirate and sputum specimens of 197 patients suspected of pulmonary tuberculosis.

Patients Inclusion and Exclusion Criteria

Children under eighteen years old attending Academic charity hospital, the reference tuberculosis laboratory and Elsha'ab Teaching Hospital, and having fever, night sweats, cough for more than 3 weeks with sputum, loss of appetite, loss of weight, chest pain and radiological evidence of tuberculosis were included.

Those cases that are over eighteen years old were excluded. Those children who do not return to reading of TST result were also excluded.

Sample Collection

Early morning gastric aspirate samples were collected from children under five years, and sputum samples were collected from the other children. Samples were collected in clean, sterile, leak-proof, wide-mouth containers.

At the time of sample collection, a Proforma was used to collect data about the patients. On obtaining the study results, the data was completed and analysed statistically using $\chi 2$ and p-value.

The processing of the samples were carried out in a biosafety cabinet. Each sample was processed by the Petroff's method and subjected to Ziehl-Neelsen (ZN) staining, Fluorescent Auramine-O (AO) staining and culture on modified Lowenstein-Jensen medium.

Slide Smear for Ziehl-Neelson Stain

Smears were fixed by gentle heat, and then flooded with carbol fuchsin and heated till steam raised, allowed to cool for 5 minutes, then washed with water and decolourized with 20% sulphuric acid, washed with water again before counter stained with methylene blue for 15-20 seconds. Smears are examined using a light

microscope scanning at least 100 oil immersion fields before reporting a smear as negative (Forbes *et al*., 1998).

AFB stain bright pink to red, beaded or barred forms are seen in Mycobacterium tuberculosis while the tissues cells and other organisms are stained blue.

Culture

Lowenstein-Jensen media were prepared. After processing, 0.25ml of the sediment from the decontaminated sputum specimen was inoculated on to the surface of two modified LJ media6. After spreading the inoculum over the surface of the slant,the tubes were incubated at 37 °C and left in the slanted position for 2 days to permit even distribution of the inoculum over the entire surface of the medium. The tubes are then placed upright and incubation continued for 8 weeks. A negative report was given if no growth appeared after 8 weeks (Cruickshank *et al*., 1975).

DNA Extraction by Phenol Chloroform Method

According to (Jain *et al*., 2002) 200µl of decontaminated sample was placed in boiling water bath at 100°C for 10 min. It was followed by incubation at 56°C for 3 hours after addition of equal amount of lysis buffer (Tris 10mM, EDTA 2mM, NaCl 0.4M and Triton X-100 0.5%) (pH 8.0) and 10µl of Proteinase K (10mg/ml). The sample was then vortexed and boiled at 100°C for 10 minutes to inactivate proteinase K. DNA purification was done by addition of equal volume of Phenol: Chloroform (24:1) followed by chloroform only. The aqueous phase was finally transferred in 2.5 volume of chilled ethanol and sodium acetate (0.3M final concentration.) was added. Tubes were kept at -20°C overnight. The sample was centrifuged at 10,000 rpm for 10 minutes and the DNA pellet was washed with 70% chilled ethanol by centrifugation. The pellet was allowed to air dry and finally suspended into 25µl of D.W. (sterile) for PCR analysis.

Primers of Insertion Sequence *IS6110*

The DNA sequence most frequently used to detect M. tuberculosis has been the insertion element IS6110 (Eisenach *et al*., 1990). Primers amplify a target fragment of 123 base pairs from the insertion-like M. tuberculosis sequences element IS6110, having the following sequence:

IS6110-F (5′-CTCGTCCAGCGCCGCTTCGG-3′)

IS6110-R (5′-CCTGCGAGCGTAGGCGTCGG -3′)

22 µl of master mix was prepared for one reaction using VIVANTIS kit (VIVANTIS Co., Ltd., Selangor Darul Ehsan, Malaysia) according to (David *et al*., 2006), as follow:

2.5 µl of 10x buffer was placed in sterile eppendorf tube (1x), 0.3 µl from 10mM forward primer was added (0.12mM), 0.1 µl from each dNTP 50 mM (0.2 mM), 1.5 µl of 25mM MgCl2 (1.5mM), 0.125 µl of 500 units at 5U/µl Taq polymerase (2.5 units), 0.3 µl from 10mM reverse primer (0.12mM), the volume was completed to 22 µl by adding 16.875 µl of sterile distilled water, the contents of master mix was vortexed after addition of each item and lastly 3µl of template DNA was added.

In negative control 3µl of sterile distilled water was added, while DNA extracted from M. tuberculosis strain H37R was used as positive control.

PCR Amplification

The reaction mixtures were then put in the thermal cycler (CONVERGYS® ltd Peltier Thermal Cycle) that carried out the following PCR program: initial 5 minutes denaturation step at 94°C for one cycle followed by repeating cycles of denaturation (30 seconds at 94°C), annealing (45 seconds at 58°C) and extension (40 seconds at 72°C) for 35 cycles, followed by a 5 minutes final extension step at 72°C (Peres *et al*,. 2009). The PCR product was visualized on 1.5% agarose gel under UV using transilluminator.

RESULTS

Out of 197 patients, 18, 33 and 35 cases were found to be positive for tuberculosis by ZN staining, culture and PCR techniques respectively. The positivity rate for ZN staining, culture and PCR in this study was 9.1% (18/197), 16.8% (33/197) and 17.8% (35/197) respectively. The

combined positivity using laboratory techniques (ZN, PCR and culture) was 7.1% (14/197).

Table 1 show that scores are definitely higher by 35 PCR positive as against 16 positive by ZN stain while table 2 shows the comparison between PCR and culture method

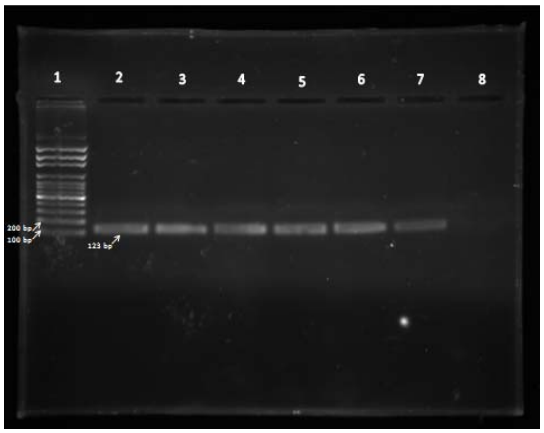

Fig. 1: Agarose gel electrophoresis of IS6110 based Polymerase chain reaction for detection of M. tuberculosis from clinical specimens. Lanes: 1 = 100 bp molecular marker; 2 = positive control (123 bp); 3,4, 5, 6, & 7 = positive from direct PCR using clinical specimens; 8 = negative control.

Table 1**:** Shows correlation between PCR and ZN stain method.

			ZN		
			positive	negative	Total
PCR	positive	Count	14	21	35
		% of Total	7.1%	10.7%	17.8%
	negative	Count	2	160	162
		% of Total	1.0%	81.2%	82.2%
Total		Count	16	181	197
		% of Total	8.1%	91.9%	100.0%

Table 2: Shows correlation between PCR and Culture on LJ media

			CULTURE		
			positive	negative	Total
PCR	positive	Count	32	3	35
		% of Total	16.2%	1.5%	17.8%
	negative	Count	0	162	162
		% of Total	.0%	82.2%	82.2%
Total		Count	32	165	197
		% of Total	16.2%	83.8%	100.0%

DISCUSSION

Many promising advances have been made in the development of novel tools to diagnose tuberculosis in adults (Brock *et al*., 2006) but none of these tests are currently in position to replace microscopy or culture (Perkins *et al*., 2006). Few of these novel approaches have been tested in children, the group in whom the diagnostic dilemma is most pronounced. At present, the use of adequately validated symptom-based diagnostic approaches and improved access to chest radiography and anti-tuberculosis treatment seem to offer the most immediate

benefit to children in tuberculosis-endemic countries with limited resources (Marais *et al*., 2006).

Molecular diagnosis for *M. tuberculosis* in children is mainly done at referral laboratories and their value as a diagnostic tool is often debated, especially in resource-limited settings.

The main aim of this study was to determine the extent to which molecular technology characterized *Mycobacterium tuberculosis* in pediatric cases by detection of specific gene directly from gastric aspirate and sputum samples. The performance of PCR test was compared with other conventional methods used in diagnosis of tuberculosis mainly ZN and culture on LJ media (gold standard method). Previously studies shown the success of microscopy is highly variable from 22% to 96% and most authors rate it at round 60% (Querol *et al*., 1995). ZN stain is routinely used in all tuberculosis diagnostic centers in Sudan. Different smear microscopy results were achieved by (Jain *et al*., 2003) ZN 32.7%, (Githui *et al*., 1993) ZN 65%, (Ulukanligil *et al*., 2000) ZN 67.6%, (Prasanthi and Kumari, 2005) ZN 50%,

This study shown that microscopic techniques were positive in 22 (11.2%) specimens, whereas IS6110 PCR showed that 35 (17.8%) specimens were positive for *M. tuberculosis*. The difference was found that to be statistical significant ($p< 0.05$).

On the other hand, culture technique detected 32 positive out of 35 positive which detected by PCR, with different only three samples less than PCR.

One of the major achievements of this study was the efficiency and success of PCR technique in characterization of *M. tuberculosis* from gastric aspiration samples (the sample which is mainly collected for detection of childhood tuberculosis for children under six years old) which detect 5 out of 89 samples, better than culture technique which detected only two gastric samples.

In addition to advantage of PCR which is rapid (needs only one day), while culture method was time consuming (4-8 weeks) and has restricted growth conditions.

There are a few limitations in this study. First, only one gastric aspirate sample collected from non-admitted children (under six years) which minimize the chance of detection positive cases, whilst two samples collected from hospital admitted children. Second, many children didn't return back after 72 hours for reading of tuberculin skin test result, which led to exclusion of them from the study.

CONCLUSION

PCR technique is more efficient over conventional methods in diagnosis of childhood tuberculosis, especially from gastric aspirate samples as it revealed rapid, accurate, specific and sensitive results

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ARABIC SUMMARY

الكشف المتطور للمتفطرة السلية لدي الأطفال السودانين باستخدام التسلسل ألإدراجي *IS 6110*

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أجريت هذه الدراسة بولاية الخرطوم في الفترة من يناير ٢٠١٠ إلى ديسمبر ٢٠١٣ للكشف عن المتفطرة السلية في الأطفال الذين يعانون من إعراض مرض الدرن باستخدام وسائل تشخيص تقليدية ومتطورة مختلفة. مائة وسبعة وتسعين عينة من غسيل المعدة والقشع تم جمعها من مستشفيات مختلفة بولاية الخرطوم.

تمت عملية إزالة التلوث من جميع العينات ومن ثم تم تزريعها بوسط لونستن جونسن بناء على طريقة بتروف المعدلة، تم إعداد مسحتين من كل عينة وتم صبغهما بصبغة زيل نلسون كما تم استخلاص الحمض النووي للجرثومة بواسطة طريقة الفينول كلوروفورم ومن ثم تم إجراء تقنية تفاعل البلمرة المتسلسل للكشف عن جين المتفطرة السلية.

أظهرت الدراسة نتائج ايجابية لفحص صبغة زيل نيلسون، طريقة التزريع و تفاعل البلمرة المتسلسلة بنسب كآلاتي ١٦ (٨.١%) ، ٣٢ (١٦.٢%) و ٣٥ (١٧.٨%) علي التوالي. خلصت الدراسة إلي ان تقنية تفاعل البلمرة المتسلسل هو الأكثر فاعلية، حساسية وتخصصية للكشف السريع عن المتفطرة السلية بعينات غسيل المعدة والقشع من الأطفال غير القادرين علي إعطاء عينة جيدة للقشع أو المشخصين بنتائج سلبية بواسطة استخدام طرق التشخيص التقليدية.