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Polymerase Chain Reaction targeting insertion sequence IS6110 for the diagnosis of pulmonary tuberculosis among Sudanese children and young adults

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ABSTRACT

This study was carried out in Khartoum State during the period from January 2011 to December 2013 to improve the rate of detection of *Mycobacterium tuberculosis* (MTB) in children with symptoms of tuberculosis (TB) infection using different conventional and advanced diagnostic techniques. One hundred and ninety-seven specimens of gastric lavage and sputum were collected from different hospitals in Khartoum State, including Elbolok Hospital, Jafar Ibn Owf Hospital, Elasha'ab Teaching Hospital, Soba University Hospital and Academy Charity Hospital.

All children participating in the study were subjected to the Mantoux test after obtaining appropriate consent injected by 5 tuberculin units of tuberculin purified protein derivative, and the results were recorded after three days. Specimens were decontaminated and inoculated on Lowenstein–Jensen media according to the modified Petroff's method. Two smears were prepared and stained by Ziehl–Neelsen stain and Auramine fluorescent dye; 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 MTB in these specimens. This study showed that the positive results for TST, ZN, Auramine, Culture and PCR were 86 (43.7%), 16 (8.1%), 22 (11.2%), 32 (16.2%) and 35(17.8%), respectively. The study concluded that the PCR technique is the most sensitive and specific technique for a quick identification of MTB in gastric lavage and sputum from children who are unable to expectorate a good quality sputum sample or who are diagnosed as negative using conventional diagnostic methods.

Introduction

Tuberculosis (TB) is a chronic contagious disease which has a major impact on global public health problems. The disease is caused by an obligate aerobic intracellular bacillus called *Mycobacterium tuberculosis* (MTB).

TB kills approximately 2 million people each year, and in 1989, the World Health Organization (WHO) estimated that

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approximately 300,000 children under 15 years of age die of TB per year worldwide [1]. Pediatric TB diagnosis is impeded by the difficulty of obtaining sputum samples from children and the paucibacillary nature of their disease that often necessitates invasive procedures such as gastric aspiration or bronchoscopy [2]. Recently, it has been shown that induced sputum has comparable diagnostic sensitivity to gastric aspirate in HIV-positive and -negative children, whereas some other studies have shown less promising results for induced sputum [3]. Although less invasive than gastric aspirates, induced sputum is still unpleasant and requires precautions to prevent airborne TB transmission to staff and other patients [4]. The important issues and challenges in pediatric TB differ markedly between developed and developing countries. In more developed countries, rates of tuberculin skin test (TST) reactivity in the general population are low, so the TST is a useful diagnostic test for TB. In contrast, in many developing countries, interpretation of the TST for diagnosis of pulmonary TB is less reliable. The diagnostic yield of gastric aspiration ranges from 20% to 40% [5].

The diagnosis of childhood TB is complicated by the absence of a practical gold standard, as bacteriologic confirmation is rarely achieved due to the predominantly paucibacillary nature of childhood TB. Sputum microscopy, often the only test available in endemic areas, is positive in less than 10%–15% of children with probable TB and culture yields are usually low (30%–40%) [6].

Specifically regarding the diagnosis of TB in children, this relies on a careful and thorough assessment of all the evidence derived from a careful history, clinical examination and relevant investigations, e.g., TST, chest radiograph and sputum smear microscopy. Although bacteriological confirmation of TB is not always possible, it should be sought whenever possible, e.g., by sputum microscopy in children with suspected pulmonary TB who are old enough to produce a sputum sample. A trial of treatment with TB medications is not generally recommended as a method to diagnose TB in children. New, improved diagnostic tests are urgently needed [7].

Objectives

General objective

To improve the detection of MTB infection among children under 18 years old in Khartoum State using IS6110.

Specific objectives

1. To identify MTB as an acid fast organism using Ziehl–Neelsen (ZN) stain.
2. To recognize MTB using Auramine stain as the best staining technique for the detection of TB.
3. To isolate MTB from gastric lavage and sputum samples.
4. To determine the best method for extraction of DNA from gastric lavage samples.
5. To characterize MTB in gastric lavage samples using the PCR technique.

Materials and methods

Type of study

Study approach

The study is a qualitative study, aimed to highlight the importance of using molecular techniques in the detection of childhood TB.

Study design

A cross-sectional laboratory-based study was conducted by collecting gastric aspirates and sputum samples from children with clinical manifestations of pulmonary TB during a six–month period from 25 May 2011 through 25 December 2011.

Study area

Five TB clinics located in Khartoum State were included in the study. Two of these TB clinics provide services to children only (Elbolok Hospital and Jafar Ibn Owf Hospital), while the other three clinics (Elasha'ab Teaching Hospital, Soba University Hospital and Academic Charity Hospital) provide services for both adults and children.

Study population

One hundred and ninety-seven children less than 18 years old suspected of having TB who attended the above-mentioned hospitals were included in the study.

Diagnostic methods

Tuberculin skin test

Tuberculin skin test (TST) was performed on all children using the Mantoux test with intradermal injection of 5 tuberculin units of tuberculin purified protein derivative (Statens Serum Institute [SSI] tuberculin RT23 in 0.1 ml solution for injection) into the volar aspect of the forearm using a 27-gauge needle. Results were read at 48–72 h and recorded as the transverse diameter of palpable induration.

Sample collection

Collecting an adequate sample presents a significant challenge, particularly in small children who cannot produce a good sputum specimen [8]. In young children (less than six years of age), early morning gastric lavage samples were collected by a nurse or physician who had been previously trained in the protocol for collecting gastric aspirates. A silastic nasogastric tube was placed, usually without sedation, before the child had taken anything by mouth. At least 5 mL of the patient's gastric secretions were collected by aspiration. When the initial attempt to aspirate was unsuccessful, 10–20 mL of sterile distilled water were instilled and aspirated. Specimens were delivered immediately to the laboratory in sealed biohazard bags in a safely closed container.

Sputum samples were collected from children more than seven years of age in clean, wide-mouthed and leak-proof specimen containers. Each patient was given a new sputum

container and clearly instructed on: how to produce an adequate sputum specimen, the importance of sputum examination for diagnosis of TB, how to open and close the containers, and the need for collecting real sputum, not saliva.

Decontamination of samples

Samples were decontaminated using modified Petroff's method by transferring to a 50-ml disposable plastic screw-capped tube and adding an equal amount of 4% of sodium hydroxide for decontamination, vortexed and then allowed to stand at room temperature for 20 min. The specimen was then centrifuged in a cold centrifuge at 3000 rpm for 15 min; the supernatant was discarded and the deposit was washed with sterile distilled water two times, and inoculated in three slopes of LJ medium. The specimen that was treated with CPC reagent was transferred to 50-ml screw-capped centrifuge tubes; sterile distilled water was filled until it reached the brim of the tube, capped and then centrifuged at 3000 rpm for 20 min. Two or three drops of the sediment were inoculated onto the three LJ slopes according to Smithwick et al. [9].

Two slide smears were prepared from the sediment for Ziehl–Neelsen stain and Auramine stain; the remaining was used for the extraction of DNA and molecular diagnosis.

Culture method

Three tubes of Lowenstein–Jensen (LJ) medium were labeled with patient number and date, and then inoculated with 20 μl of the sediment that was obtained from the digestion and decontamination of the sputum sample. Two of the three tubes contain glycerol while the third tube contains pyruvic acid to isolate the *Mycobacterium bovis* species if present. All cultures were incubated at 37 °C in slope position for three days with loosened caps to evaporate the excess fluid, then the caps were closed tightly to prevent drying of media, bottles were placed in an upright position and examined for contamination. 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 finally every week to detect slow growers up to the eighth week.

Ziehl–Neelsen stain

Two drops from the sediment of decontaminated sputum were placed on a clean and dry microscope slide and spread out. The smear was left to dry completely and fixed by passing carefully through the flame three times. The proper temperature for flame fixation was checked by touching the slide to the back of the hand immediately after removing it from the flame. The smear was covered with carbol fuchsin and heated gently until a vapor raised; the heating of the stain was allowed for 5 min and then washed off with clean water. Decolorization was done by using 25% sulfuric acid for a few seconds. Washed and lastly, methylene blue was added for 2 min. Thereafter, it was washed, dried and examined under a microscope. Positive result shows red bacilli with a blue background, while negative results show a blue background with no red bacilli.

Auramine stain

The smear was flooded with Auramine solution and allowed to stain for 10 min. Then the smear was rinsed and drained; acid alcohol was used to de-stain for 2 min, the smear was rinsed again in distilled water and drained. Finally, the smear was flooded with potassium permanganate for 10 s, followed by several rinses with distilled water. The back of the slide was wiped clean and placed in a draining rack for drying.

Smears were examined systematically for AFB by fluorescent microscope (Olympus BX51) using the 40× objective. Positive results show fluorescent apple green rods glowing against a dark background.

Molecular identification

Nucleic Acid Amplification Techniques (NAAT) are diagnostic methods based on the amplification of MTB DNA. Polymerase Chain Reaction (PCR) was used as a rapid and accurate diagnostic tool for detection of pulmonary TB in children.

Phenol chloroform method

According to Jain Amita [10] 200 μl of decontaminated sample was placed in a boiling water bath at 100 °C for 10 min. It was followed by incubation at 56 °C for 3 h after the addition of equal amounts of lysis buffer (Tris 10 mM, EDTA 2 mM, NaCl 0.4 M and Triton X-100 0.5%) (pH 8.0) and 10 μl of proteinase K (10 mg/ml). The sample was then vortexed and boiled at 100 °C for 10 min to inactivate the proteinase K. DNA purification was done by the 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 (0.3 M final concentration) was added. Tubes were kept at −20 °C overnight. The sample was centrifuged at 10,000 rpm for 10 min, and the DNA pellet was washed with 70% chilled ethanol by centrifugation. The pellet was allowed to air dry and finally suspended into 25 μl of D.W. (sterile) for PCR analysis.

Primers of insertion sequence IS6110

The DNA sequence most frequently used to detect MTB has been the insertion element IS6110 [11]. Primers amplify a target fragment of 123 base pairs from the insertion-like MTB sequences element IS6110 with the following sequence:

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

Preparation of master mix

Before starting the master mix preparation, the hood was disinfected using 70% ethanol before and after the preparation of each batch, then sterilized further by turning on the UV light for at least 20 min.

22 μl of the master mix was prepared for one reaction using VIVANTIS kit (VIVANTIS Co., Ltd., Selangor Darul Ehsan, Malaysia) according to David [12], as follows:

2.5 μl of 10× buffer was placed in a sterile eppendorf tube (1×), 0.3 μl from 10 mM forward primer was added (0.12 mM), 0.1 μl from each dNTP 50 mM (0.2 mM), 1.5 μl of 25 mM $MgCl_2$

(1.5 mM), 0.125 μl of 500 units at 5 U/μl Taq polymerase (2.5 units), 0.3 μl from 10 mM reverse primer (0.12 mM), the volume was completed to 22 μl by adding 16.875 μl of sterile distilled water, the contents of the master mix were vortexed after the addition of each item and lastly 3 μl of template DNA was added.

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

PCR amplification

The reaction mixtures were then placed in the thermal cycler (CONVERGYS® ltd Peltier Thermal Cycle) that carried out the following PCR program: initial 5 min denaturation step at 94 °C for one cycle followed by repeating cycles of denaturation (30 s at 94 °C), annealing (45 s at 58 °C) and extension (40 s at 72 °C) for 35 cycles, followed by a 5-min final extension step at 72 °C [13].

Loading of samples and electrophoresis

8 μl of PCR product from each sample were mixed with 2 μl of loading dye and then the mixtures were delivered into the well. 7 μl of DNA ladder (marker) length 100 bp ladder with fragments ranging from 100 bp to 1000 bp were added to one well in each run to estimate the size of tested DNA sequence. The gel electrophoresis apparatus was connected to a power pack (Serva BluePower 500, Germany). The electrophoresis was performed at 50 V for 30 min.

Visualization of PCR product

After the electrophoresis period, the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. One hundred and twenty-three base pair (bp) target DNA fragments, specific for MTB complex, were viewed under ultraviolet transilluminator (SYNGENE, UK). Lastly, the gel was transferred to gel documentation system (Olympus) for photography documentation.

Results

Epidemiological finding

Gender
During the six-month study period, 197 children of both sexes with a clinical manifestation of TB were enrolled in the study; 71 (36%) were females and 126 (64%) males giving an average sex ratio of 1:1.8.

Age group
The median age at investigation was 8 years. The study population was divided into three age groups according to the ability to expectorate a sputum sample; 86 (43.7%) children less than 6 years old who cannot give a good sputum sample, were candidates for the collection of a gastric aspirate. 63 (32%) children between the ages of 7 and 12 years old, a gastric aspirate was collected from those who could not produce a sputum sample. The last group of 48 (24.3%) children, all of them produced a good sputum sample. Of the 197 samples, there were 89 (45.2%) gastric aspirates, while 108 (54.8%) were sputum samples.

Exposure to risk factor and disease manifestations

Contact with an adult with TB was reported for 61 children (31.0%), and 173 children (87.8%) had a cough lasting more than two weeks. Weight was recorded as being less than 40% of expected weight-for-age according to Ministry of Health – Primary Health Care standard in 166 (84.3%) cases. Of the 197 children studied, there were 174 (86.6%) patients suffering from fever. Among the participating children, there were no HIV positive cases.

Laboratory findings

TST
Tuberculin skin test (TST) was performed for 201 children; 4 children did not return after two days for the result reading and were excluded from the study. 197 children were enrolled in the study. Of the 197 children enrolled in the study, 86 (43.7%) tested positive for TST.

ZN stain
Of the 197 specimens studied that were slide-smear prepared, 16 (8.1%) tested positive for ZN stain. All positive results were found to be from sputum samples.

Auramine stain
Of the 197 specimens studied, 22 (11.2%) tested positive for Auramine stain. All positive results were found to be from sputum samples.

Isolation

During the period of the study, 204 specimens were subjected from the above-mentioned hospitals. All of these samples were decontaminated and cultured on LJ media. Out of these, 7 specimens were found to be contaminated. 197 specimen results were used in the study. Out of the 197 specimens, 32 (16.2%) showed slow growth MTC-like colonies, 2 (1.01%) showed rapid growth and were considered as negative for MTB. Of the 32 slow growth MTC-like colonies, there were 30 (93.8%) organisms isolated from the sputum samples, while 2 (6.2%) were isolated from the gastric aspirate. The MTC-like colonies were confirmed by conventional methods.

Growth rate

The growth rate of the isolates ranged between 3 days and 6 weeks. Most of the isolates showed visible growth after 3 weeks. Two out of 197 isolates were identified as rapid growers of mycobacteria, while the growth rate of 32 isolates ranged between 4 and 6 weeks; they were identified as slow growers and tentatively considered as belonging to the MTC species. The rest of the 163 cultures (83%) showed no growth.

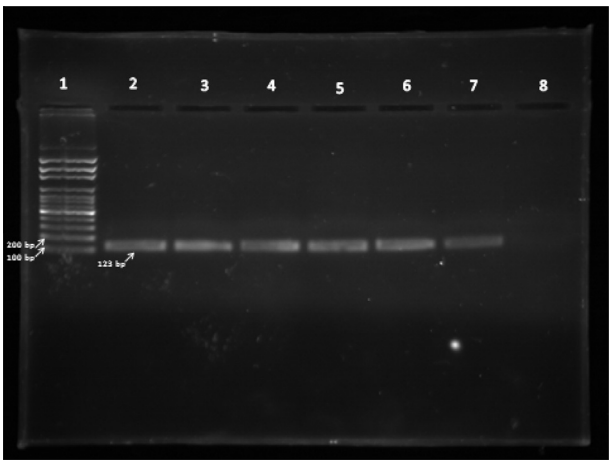

Fig. 1 – Agarose gel electrophoresis of IS6110 based polymerase chain reaction for detection of *M. tuberculosis* from clinical specimens. Lanes: 1 = 100 bp molecular marker; 2 = positive control (123 bp); 3–7 = positive from direct PCR using clinical specimens; 8 = negative control.

Cultural characteristics

Cultural properties of all 32 slow-growing isolates of MTB complex on Lowenstein–Jensen medium at 37 °C were almost the same, and all colonies were found to be dry, rough and (buff) colored.

Polymerase Chain Reaction

From the 197 specimens that were directly subjected to PCR, 35/197 (17.8%) showed a band typical in size (123 bp) to the target gene (IS6110) as indicated by the standard DNA marker (Fig. 1); 162/197 (82.2%) samples were negative. Out of 35 positive results, 4 (11.4%) were yielded from gastric aspirate samples, while 31 (88.6%) were yielded from sputum samples.

Sensitivity and specificity

The sensitivity and specificity of TST, microscopic techniques and PCR based on results of culture as gold standard techniques for the diagnosis of TB were as follows: 100%, 59.38%, 43.75% and 100% sensitive for TST (Fig. 1), Auramine, ZN and PCR test, respectively. While 67.27%, 98.18%, 89.79% and 98.18% showed specificity. Positive Predictive Value (PPV) was 37.23% for TST, 86.36% for Auramine and 87.50% for ZN stains, and 91.43% for PCR. Negative Predictive Value (NPV) was 100%, 92.57%, 90.06% and 100%, respectively.

Discussion

Childhood TB is often regarded as unimportant in the epidemiology of TB because >95% of children with TB are sputum-smear negative and therefore do not contribute to the immediate spread of the disease. Childhood TB, however, is a marker for ongoing transmission of infection within a community, and infected children represent the pool from which a large proportion of future cases of adult TB will arise [14].

Furthermore, TB is an important cause of morbidity and mortality in children. The World Health Organization estimated that 1.3 million cases of TB and 450,000 deaths from TB occur annually among children in developing countries [15].

This study showed that the frequency of childhood TB diagnosis using PCR technique is 17.8%, and 16.2% using the culture technique that is similar to the results of published studies in the nearby countries of Ethiopia 16.1%, Nigeria 12.4%, and Tanzania 16.1% [16].

Many promising advances have been made in the development of novel tools to diagnose TB in adults [17], but none of these tests are currently in position to replace microscopy or culture [18]. 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 TB-endemic countries with limited resources [19].

Most recently developed sensitive and specific diagnostic tests have not found a place in the routine evaluation of children with suspected TB. Clinical criteria, particularly skin-test results, radiographic changes, and documented exposure to an infectious adult remain standard diagnostic methods.

Molecular diagnosis for MTB 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 is to determine the extent to which molecular technology characterized MTB in pediatric cases by detection of a specific gene directly from gastric aspirate and sputum samples. The performance of the PCR test was compared with other conventional methods used in the diagnosis of TB TST, ZN, Auramine and culture on LJ media (gold standard method).

Despite the low specificity of TST particularly in children, the test is widely used in the diagnosis of childhood TB. In this study interpretation of TST results is depended on the Centers for Disease Control and Prevention (CDC) recommendations regarding the size of the induration created by the TST that is considered a positive result and indicative of disease. The TST is interpreted on the basis of three cutoff points: 5 mm or more is considered a positive TST result for children with close contact to a known or suspected contagious case of the disease, including those with household contacts with active TB whose treatment cannot be verified before exposure. 10 mm or more is considered a positive TST result for children who are at a higher risk of dissemination of TB disease, including those younger than 5 years or those who are immunosuppressed because of conditions such as malnutrition. Induration of 15 mm or more is considered a positive TST result in children aged 5 years or older without any risk factors for the disease.

TST was found to have the highest positive result of 86/197 (43.7%) among all diagnostic methods used in the study. There are two possible explanations for the high TST results compared with other investigations. First, false-positive TST reactions due to BCG vaccination in young children–the vast majority of children enrolled in the study were less than

10 years old. In a review of many published studies regarding the effect of BCG vaccination on TST, only 1% of subjects vaccinated as infants were TST-positive if tested ⩾10 years after being vaccinated by BCG [20]; second, false-positive due to infection by environmental non-TB mycobacteria (due to cross-reactivity).

Previous studies showed the success of microscopy is highly variable from 22% to 96% and most authors rate it at round 60% [21]. ZN stain is routinely used in all TB diagnostic centers in Sudan, while Auramine fluorochrome method is only used in the National Reference Laboratory (NRL). Different smear microscopy results were achieved by Jain [22]; ZN 32.7%, Auramine 41.6% [23], ZN 65%, and Auramine 80% [24]; ZN 67.6%, Auramine 85.7% [25], ZN 50%, and Auramine 69%. It was evident that Auramine method results scored higher than that of the ZN method in all these studies as was the case in this study (ZN 8.1%, Auramine 11.2%). In this study, Auramine was found to be 3.1% more effective than ZN staining. This shows that fluorochrome staining of sputum smears in comparison with that of ZN staining is a better method of microscopy.

The poor performance of conventional MTB detection techniques, based on microscopic examination of Ziehl-Neelsen stained, culture of MTB on LJ medium and TST are still in widespread use for diagnostic purposes, though they still fail to provide the required sensitivity and specificity. The PCR test would be particularly useful in the diagnosis of childhood TB where conventional microbiological techniques for MTB are showing poor performance of sensitivity and specificity.

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

On the other hand, culture techniques detected 32 positive out of 35 positive which was detected by PCR, with only a three sample difference less than PCR.

One of the major achievements of this study was the efficiency and success of PCR technique in characterization of MTB from gastric aspiration samples (the sample which is mainly collected for the detection of childhood TB for children under six years old), which detected 5 out of 89 samples, better than the culture technique which detected only 2 gastric samples.

In addition, the advantage of PCR is that the results are rapid (needs only one day), while culture methods are time-consuming (4–8 weeks) and have restricted growth conditions.

There are a few limitations in this study. First, only 1 gastric aspirate sample was collected from non-admitted children (under 6 years), which minimizes the chance of detecting positive cases whilst 2 samples were collected from hospital-admitted children. Secondly, many children did not return back after 72 h for reading of the TST result, which led to the exclusion of them from the study.

Conclusions

PCR technique is more efficient over conventional methods in the diagnosis of childhood TB, especially from gastric aspirate samples.

IS6110 PCR test for DNA-specific MTB may be a hope for the rapid and accurate diagnostic test for childhood TB, and it will help where conventional diagnosis fails and provisional diagnosis of childhood TB is made on the basis of clinical presentation without evidence of AFB. IS6110 PCR may have a great potential to improve the clinician vision for the early diagnosis, treatment and prevention of childhood TB.

The specificity, sensitivity and speed of the PCR test in the diagnosis of MTB infection shown in this study should encourage the use of this method in routine diagnosis of pediatric TB.

Auramine stain performs better than ZN stain in detecting TB bacilli in sputum, particularly the paucibacillary cases. Since screening is done under a lower power of magnification (40×), fluorescence microscopy has been found to be less time-consuming as compared with the ZN method (100×) in the diagnosis of TB. Hence, it has been advocated to be a method of choice where a large number of sputum smears are to be examined. The fluorescing bacilli are easily identifiable and cause less eye-strain.

TST is a highly sensitive method, but specificity is affected by recent BCG vaccination, whereas the majority of participants in this study were young children.

Conflict of interest

The authors have no conflict of interest to declare.

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