Use Of PCR Amplification For The Evaluation Of TB Diagnosis In Children By Classic Methods.

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الخلاصة

توجد دراسات قليلة حول قياس كفاءة تقنية الـ PCR مقارنة بالفحوص الروتينية لتشخيص مرض التدرن الرئوي في الاطفال, اذ ان فحص التيوبركلين الجلدي المستخدم في الكشف عن الاصابة بعصيات TB في الاطفال اخذ بالتراجع مما يتطلب البحث عن تقنية جديدة.
في هذا البحث تم قياس نواتج التشخيص للـ PCR لعينات من مرض الاطفال الذين راجعوا مركز الامراض الصدرية في الناصرية للمدة من كانون الثاني الى ايار 2008 ومقارنة النتائج مع الفحوصات الروتينية مثل فحص التيوبركلين الجلدي والتصبغ بصبغة زيل – نلسن والزرع على وسط لونشتاين – جنسن الذي اعتمد كاختبار اساسي للتشخيص . جمعت 108 عينة قشع لمرضى شخصوا بالاصابة بالـ TB. اظهرت النتائج ان فحص التيوبركلين الجلدي اعطي 23(21.3%) شحنة موجبة بنسبة حساسية 65.7%ونوعية 87%بالمقارنة مع 35(32.4%) للزرع المختبري . اما فحص الـ PCR فكان نسبة حساسيته 88.6% والنوعية 100% وهو اكثر بالمقارنة مع الفحوصات الاخرى .نستنتج من هذا ان تقنية الـ PCRهي الاكثر ملائمة مقارنة بالفحوصات الروتينية الاخرى المستخدمة في تشخيص الـ TB عند الاطفال.

Abstract

There have been few studies evaluating the efficacy of polymerase chain reaction (PCR) testing in Front other routine test for diagnosis of TB in children. The tuberculin skin test (TST) used to detect *Mycobacterium tuberculosis* infection in children has many drawbacks, and a new diagnostic test for has been introduced. We assessed the diagnostic yield of PCR prospectively in a blinded study of patients referred to Al-Nassyria center for chest diseases and other private laboratories from January to June 2008 and compared the results to the routine TST, smears with Ziehl-Neelsen staining and culture on Lo¨wenstein-Jensen medium which used as the "gold standard.". Sputum specimens were collected from 108 TB suspects patients and analyzed for the presence of *Mycobacterium tuberculosis..TST yielded* 23 (21.3%) *were positive for TB with* 65.7 % sensitivity and 87% specificity in comparing with 35(32.4%) by culture which improved the presence of 3cases as false-positive and 15 cases as false-negative by TST .

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All smear positive specimens appeared culture positive, but showed 54.3 %sensitivity and 100 % specificity. PCR amplification results appeared less than culture and more than each of smear and TST with %88.6 sensitivity and 100% specificity .All the thirty one PCR positive samples showed culture positive. It is concluded that although both culture and PCR amplification method are Sensitive and specific for the detection of *M. tuberculosis* in respiratory specimens of children . Rapid PCR method appeared to be more suitable than other tests especially the routine TST which applied.

Introduction

Tubercal bacilli(TB) still an important public health problem worldwide, it is one of the leading infectious disease in the world and is responsible for more than 3 million deaths. Globally, the burden of tuberculosis is immense with an estimated one third of the world's population infected and projections that almost 12 million new cases will occur annually by 2006[1]. *Mycobacterium tuberculosis* is the most common cause of T.B, other rare causes are *M. bovis* and *M. africanum*[2]. It is necessary to find method for rapid diagnosis of this disease. Microscopic detection of the causative organism on stained smear samples processed for clinical specimens could provide quick results but its specificity and sensitivity is not enough [3].

The classic diagnostic tool for latent tubercal bacilli infections (LTBI) is the tuberculin skin test (TST), also known as the intradermal Mantoux test since 1910. It is the oldest diagnostic test in use in modern medical practice, and its limitations constitute the weakest element in the strategy of targeted testing of LTBI [4]. Thus, the poor sensitivity of TST has a negative effect on the management of individuals who would benefit the most from targeted testing and preventive treatment[5]. This same limitation of the skin test applies to its use as a diagnostic aid in the evaluation of cases of suspected active TB, when microbiological confirmation is awaited or not possible. However, due to its poor sensitivity, a negative TST in these patients is almost invariably clinically unhelpful and therefore not recommended by current guidelines [6].The main drawback with the clinical use of the TST is the lack of specificity due to cross-reactivity with proteins present in other mycobacteria, such as the *Mycobacterium bovis* bacillus Calmette-Gue´rin (BCG) vaccine strain and *Mycobacterium avium* [5].

Early diagnosis of infectious cases and treatment of individuals latently infected with *Mycobacterium tuberculosis*, who are at

increased risk of progression to active disease, are the key strategies for reducing the incidence of TB in low-prevalence areas[4,7]. Despite these limitations, the TST is routinely used in hospital clinical practice for the screening of LTBI [5], and as a diagnostic tool for active disease in children in Iraq.

PCR is a well-developed technique used extensively for the diagnosis of numerous infectious diseases, including tuberculosis. This method based on DNA amplification and hybridization and used for the rapid detection of *Mycobacterium tuberculosis* [8].Many amplification targets for *M. tuberculosis* have been reported. One of the more common PCR targets is the repetitive sequence IS6110, specific for *M. tuberculosis* complex [9]. An alternative approach utilizes the FDA-approved Roche Amplicor *M. tuberculosis* kit, which targets the 16S ribosomal RNA gene for amplification with subsequent detection of the products using an *M. tuberculosis* specific probe[10]. Developments in this area have been very rapid and a large number of PCR assays targeting different gene stretches of *M. tuberculosis* have been described .

We performed this prospective study to compare utility of PCR techniques with that of AFB smears, tuberculin test and culture for diagnosis of pulmonary tuberculosis in children.

Materials and Methods

Patients: A total of 108 patients of children (8-16 years old) from both genders referred to Al-Nassyria center for chest diseases and other private laboratories from January to June 2008 , with a high clinical suspicion of localized forms of pulmonary tuberculosis and most of them suffering from fever, weight loss, cough with bloody sputum and anorexia and not receiving anti-tuberculous therapy.

Collection of sputum: Trained staff were collected the samples. Suspects were counseled to deliver three sputum specimens. The first specimen was collected when the patient attended the clinic for the first time. A container was given to the patient for collecting an early morning sample at home the next day, and a third was collected again at the clinic when the patient brought back the early morning specimen. Normal saline induction was performed for patients unable to expectorate spontaneously. Specimens were decontaminated by the *N*-acetyl-L-cysteine-NaOH method and concentrated by centrifugation [11]. Portion of the pellet was frozen at zero degrees centigrade and then transported to the molecular biology laboratory – College of Medicine / Al-Qadisia University for PCR analysis.

Bacterial culture: specimens were inoculated in duplicate onto slants of *Lowenstein-Jensen medium* (Oxoide) . The inoculated slants were incubated at 37°C and examined for growth twice weekly for the first 2 weeks and once weekly thereafter up to 8 weeks, after which a definitive result was obtained. Cultures that showed no growth after 8 weeks were scored as "negative." A patient was defined as a "TB-positive case" if one of the three sputum specimens had a positive culture and as a "non-TB case" if none of the three sputum specimens showed growth.[12].Diagnosis according to [13].

Acid-fast staining (AFS) . A Smears for microscopic examination were prepared from the concentrated specimens and stained using the Ziehl-Neelsen method. A suspect was diagnosed as a smear-positive TB patient when at least one of the three smears was positive by Ziehl-Neelsen method [11].

Tuberculin Skin Test: Administration the TST (one-step method) to the patients only according to the guidelines established by the Centers for Disease Control and Prevention [12]. Briefly, 0.1 ml of the tuberculin PPD (TUBERSOL; Aventis Pasteur Ltd., Canada) containing five tuberculin units was injected intradermally on the volar surface of inmates' forearms. After 48 to 72 h, the diameter of the area of indurations around the injection site was measured across the forearm and was reported in millimeters. Reactions of ≥10 mm were considered positive. Treatment for LTBI among the study subjects was based solely on the TST results.

PCR Assay: Reagent and sample preparation, PCR amplification, and product detection were performed in separate rooms using dedicated equipment, positive-displacement pipettors, and unidirectional work flow. In addition, dUTP was incorporated into the amplicons so that they could be inactivated by UNG nuclease to prevent carryover contamination. Positive and negative controls for both the sample preparation and PCR processes were utilized in each experiment, and they included both known positive and negative sputa as well as a dilution of tuberculosis DNA as a positive control and water blanks as negative controls. All samples also underwent amplification of a fragment of the human p53 gene as a control for amplification inhibitors[9].

The amplicator test system (Cinnagen Inc-Iran) based on repetitive sequence IS 6110 of MTP and by the application of two oligonucleotide primer IS1 and IS2 (Table 1), and for the detection of 163-bp DNA fragment. Testing was performed according to the manufacturer's instructions.

Table -1: Oligonucleotide primer sequences used

Primer	Primer sequence		Length
IS1	Forward	5'-CCTGCGAGCGTAGGCGTCGG	20
IS2	Reverse	5'-CTCGTCCAGCGCCGCTTCGG	20

Results

One hundred eight patients were tested by tuberculin skin test , acid fast stain for sputum , culture for sputum on *Lowenstein-Jensen medium, PCR amplification* in addition to chest X-ray results for establishing the diagnosis of TB.

The routine diagnostic procedure *of TST yielded from the total patients , twenty three (21.3%) were positive for pulmonary TB* .The diameter of the area of induration around the injection site was >10 mm , and the treatment was based solely on the TST results. All the infected children appeared follows discovery of a case in an adult. The patients were divided into three groups according to TST results, table 2.

Table -2: Groups of patients according to the results of TST and chest X-ray

groups		Number	%
1	TST +ve , X-ray +ve	23	21.3
2	TST –ve , X-ray +ve	15	13.9
3	No T.B	70	64.8
Total		108	100

Group number 1 includes 23 patients were appeared positive for TST and X-ray, group number 2 includes 15 patients were positive for X-ray and negative for TST and group number 3 includes 70 patients were negative for each TST and X-ray.

A comparison of routine TST results with amplification PCR , smears, cultures is summarized in table,3. Culture was considered the gold standard . Of 108 patient samples, 35 (32.4%) were positive for culture, distributed as following: 20 in group 1 ; 7 in group 2 and 8 in group 3.

Table -3: Comparison of TST with culture, smear and PCR results

Test Groups	TST %	Culture results		Z.N smear		PCR result	
		+ve %	-ve %	+ve %	-ve %	+ve %	-ve %
TST positive	23 21.3	20 87	3 13	16 69.6	7 30.4	20 87	3 13
TST negative	15 13.9	7 46.7	8 53.3	1 6.7	14 93.3	5 33.3	10 66.7
NO T.B	70 64.8	8 11.4	62 88.6	2 2.9	68 97.1	6 8.6	64 91.4
Total	108 100	35 32.4	73 67.6	19 17.6	89 82.4	31 28.7	77 71.3

TST positive appeared less in three than positive culture with 65.7 % sensitivity and 87 % Specificity (table, 4). A total of 15 patients (7 in group 2 and 8 in group 3) were negative for TST and showed culture positive.

Of 23 TST positive, only 16 patients were smear positive. In addition to other 3 positive patients from TST negative groups (1 in grouop2 and 2 in group 3), the total of smear positive were 19 . All smear positive samples appeared culture positive, and showed 54.3 %sensitivity and 100 % specificity .

A comparison of PCR amplification results with TST , smears and cultures showed that PCR results less than culture and more than each of smear and TST (table,3) with %88.6 sensitivity and 100% specificity (table, 4) . All the thirty one PCR positive samples showed culture positive, and both methods were identical in the detection of twenty positive TB in patients of group 1. PCR was detected positive TB in eleven patients (5 in group 2 and 6 in group 3) were they appeared TST negative .

Table -4 : Sensitivity and specificity of TST, smears and PCR for detection of TB in comparison with culture as gold standard .

Method	Sensitivity %	Specificity %
TST	65.7	87
smears	54.3	100
PCR amplification	88.6	100

Discussion

The diagnosis of tuberculosis in children is difficult and often delayed. Bacterial evidence of infection especially positive smears are rarely obtained[14]. Experts in the field suggest that TB control depends on rapid diagnosis and effective treatment. The clinical presentation and history can help in presumptive diagnosis [15].

Our study attempted to provide some answers to the questions of diagnostic yield of PCR prospectively in a blinded study comparing PCR results with cultures , TST and smears for diagnosis of tuberculosis. The present study corresponding with previous study [12] and revealed that TST has low sensitivity (65.7%) and specificity (87%) in comparing with culture and PCR for diagnosis of tuberculosis. TB diagnosis in children usually follows discovery of a case in an adult , and relies on tuberculin skin testing, chest radiograph, and clinical signs and symptoms [12].However, clinical symptoms are nonspecific, skin testing and chest radiographs can be difficult to interpret because chest radiograph are either normal or show only fibrotic lesions or calcifications in the lung parenchyma or regional lymph nodes, and routine laboratory tests are not helpful [16].

In addition to that ,the results showed there were three cases false-positive and fifteen cases were false-negative by TST. The false-negative rate cannot be calculated. A negative TST does not rule out TB disease in a child. Approximately 10% of otherwise normal children with culture-proven TB do not react to tuberculin initially [17,18] .False-positive reactions to TST are often attributed to asymptomatic infection by environmental nontuberculous mycobacteria (NTM), in addition vaccination with *M. bovis* can cause transient reactivity to a subsequent TST [19]. Furthermore ,the low sensitivity and specificity, TST has many drawbacks, such as the need for patients to return for test reading, as well as variability and subjectivity in test application and reading. Other studies were reported these notes [20].Because most current diagnostic tests for TB infection and disease have low specificity and therefore low positive predictive values, epidemiologic investigation continues to be important in establishing the diagnosis of TB in children.

AFB staining lacks sensitivity by detected the presence of *M. tuberculosis* in 54.3% of positive culture. The relatively low positive predictive value in smear-negative patients makes interpretation of a positive test less certain. These findings are consistent with a recent

comprehensive review [21], and the current recommendations of the Centers for Disease Control and Prevention [22].

Amplification of *M. tuberculosis* specific IS6110 in clinical specimen is appear the most sensitive method after culture and there is no significant value between both methods ,. These was in accordance with the findings of previous studies [12,14]. A rapid initial diagnosis of *M. tuberculosis* infection is problematic if the techniques of direct visualization are negative. The definitive diagnosis depends on culture of the mycobacteria, a technique that is time-consuming . Furthermore, the performance of inconvenient invasive procedures is often necessary, especially when dissemination is suspected, in order to obtain appropriate specimens for culture [23]. Because of the high sensitivity and specificity of the PCR improved to detect *M. tuberculosis* DNA from clinical samples and corresponding the results with other studies (8, 14, 24), we have investigated the potential use of this technique as a rapid diagnostic procedure for tuberculosis. In this study, we detected *M. tuberculosis* DNA in samples from children.

In summary, the results presented in this study confirm previous reports that indicate that a great improvement in the rapid diagnosis of tuberculosis, is possible by the direct amplification of *M. tuberculosis*, our findings indicate that PCR testing of sputum samples prove diagnostic in patients who have TST-positive tuberculosis and more than 33% of TST-negative pulmonary infections.

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