



A comparative Study of Tuberculin Skin Test with Fluorescent, Ziehl-Neelsen Staining and Culture for the Diagnosis of Pediatric Tuberculosis in Khartoum State

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ABSTRACT

Tuberculosis is one of the leading causes of mortality from an infectious disease world-wide. Current estimates indicate that more than two million people die from this disease each year. This study was carried out in Khartoum state during the period between May and December 2011. The main objective was to compare Tuberculin skin test with fluorescent, Ziehl-Neelsen (ZN) staining and culture for the diagnosis of pediatric tuberculosis. 197 samples of gastric lavage and sputum were collected from different TB centers in Khartoum State including Academic Charity Hospital, Soba University Hospital and Elshab Teaching Hospital.

Tuberculin Skin Test was done for every patient, Then ZN and Auramin O (AO) staining smear and culture were performed. The study showed that only 41.9% of Tuberculin Skin Test (TST) positive children were found to be positive with other laboratory tests (ZN, Ao stain and culture). This study concluded that TST is highly sensitive in detection of TB infection, but low specific in comparison with ZN, Auramin O and culture (gold standard for diagnosis of TB infection). The study results indicate that factors other than tuberculosis, such as NTM infection or previous BCG-vaccination, are widely contributing to positive TST results among children in Sudan.

المستخلص

مرض السل هو واحد من أهم الأمراض المعدية المسببة للوفيات في جميع أنحاء العالم، وكل الإحصائيات الحالية تشير إلى أن عدد الوفيات يصل إلى أكثر من مليوني شخص كل عام.

أجريت هذه الدراسة بولاية الخرطوم في الفترة ما بين مايو وديسمبر 2011 م ، وقد هدفت الي مقارنة اختبار السلين الجلدي مع صبغتي الأورامين الفلورية وزيل نيلسون والتزريع لتشخيص مرض السل في الأطفال.

تم جمع 197 عينة من غسيل المعدة والقشع من عدة مستشفيات في ولاية الخرطوم وهي مستشفى الأكاديمية الخيري التعليمي، مستشفى الشعب ومستشفى سوبا الجامعي.

تم إجراء اختبار السلين الجلدي لكل المرضى وبعد ذلك تم صبغ مسحات العينات بطريقة الأورامين وزيل نيلسون وزُرعت العينات لعزل بكتريا المتفطرة السلية. أظهرت الدراسة أن 41.9 % فقط من الأطفال موجبي اختبار السلين الجلدي كانوا موجبي الاختبارات المعملية الأخرى (صبغة زيل نيلسون والأورامين والتزريع). استخلصت الدراسة أن اختبار السلين الجلدي هو الأكثر حساسية في تشخيص مرض السل ولكن ذو خصوصية أقل مقارنة مع الاختبارات المعملية الأخرى (التصبغ بصبغة اورامين الفلورية وزيل نيلسون والتزريع) والذي يعتبر المعيار الذهبي في تشخيص مرض السل. كما أظهرت الدراسة أن هناك عوامل أخرى غير مرض السل مثل انواع المتفطرة الأخرى التي لا تسبب السل أو التطعيم السابق بلقاح السل قد تساهم بصورة كبيرة في ايجابية نتيجة اختبار السلين الجلدي بين الاطفال في السودان.

KEYWORD: *Tuberculin skin test, Ziehl-Neelsen stain, Lowenstein- Jensen medium, Pediatric tuberculosis.*

INTRODUCTION

Tuberculosis (TB) is a chronic contagious disease which has a major impact on global public health problem. The disease is caused by an obligate aerobic intracellular bacillus called, *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 ⁽¹⁾. TB remains a leading cause of mortality in children worldwide. The diagnosis of TB in children is traditionally based on exposure history, symptoms, the tuberculin skin test, chest radiography, and mycobacterial staining and culture. However, these investigations lack sensitivity and specificity and diagnosing childhood TB remains a major global challenge ⁽¹⁾. For over 100 years the standard diagnostic test for *M. tuberculosis* infection has been the TST. In 1882 Robert Koch boiled the culture of tubercle bacilli and injected it into people as a means to treat tuberculosis. This experiment failed, as overwhelming inflammatory responses developed and resulted in several deaths. However, what emerged from this experience was a definitive means to identify *M.*

tuberculosis infection. In 1934, an American scientist, Dr. Florence Siebert, developed a method of purifying the tuberculin and made a simple protein precipitate (purified protein derivative PPD), a solution of antigens produced by the metabolic activity of *M. tuberculosis*. Today the definitive TST uses five tuberculin units of PPD injected intradermally with the Mantoux technique ⁽²⁾. Childhood TB accounts for 6% to 10% of all TB cases worldwide; At least half a million children worldwide get sick with TB disease each year; and More than 74,000 children die from the disease each year. ⁽³⁾. Unfortunately, these figures underestimate the burden of childhood TB worldwide. TB in children has been a "hidden epidemic" for many years because of a number of challenges ⁽³⁾. Childhood TB is particularly difficult to diagnose in resource-poor settings and is often not reported to health authorities in many countries. Many children cannot cough up sputum for TB testing. Even when sputum from a child is available, the least expensive tests can diagnose only about 30% of cases ⁽³⁻⁶⁾. TB in a child represents recent and ongoing transmission of TB bacteria. Young children are most likely to become exposed and infected with TB by close

contacts, such as family members. Children can develop TB disease at any age, but the severe forms of TB are most common among children between 1 and 4 years of age. Children can get sick with TB disease very soon after being infected with TB bacteria, or they can get sick at any time later in life. They can even infect their own children, decades later, if not treated⁽³⁾. TB in adults and children is curable if identified and treated appropriately. Children at risk of developing TB disease can be identified using simple methods and screening tools. Many children with TB disease can be diagnosed with a clinical evaluation by a trained health care worker⁽³⁻⁶⁾. In this study, tuberculin method is compared with the staining methods and culture on modified Lowenstein- Jensen (LJ) medium, as “gold standard”.

MATERIALS and METHODS

Ethical Clearance

This study was approved by the National Ethics Committee, Ministry of Health-Sudan. Written consent was obtained from every patient before being enrolled in the study.

Study Design and Collection of the Samples

This is a descriptive comparative study conducted in the Department of Tuberculosis, National Health Laboratory, Khartoum, Sudan, during the period from May to December 2011, on gastric aspirate and sputum specimens of 197 patients under eighteen years old who attend 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 production, loss of appetite, loss of weight and chest pain. All enrolled patients were categorized into three age groups (Figure 1).

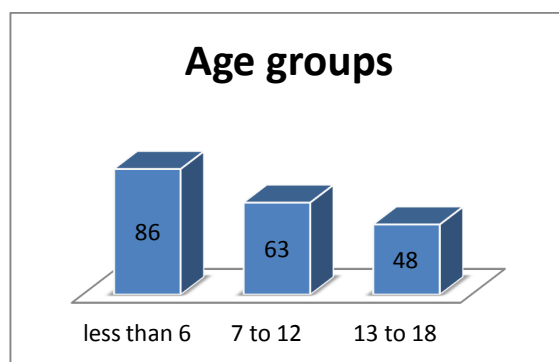


Figure 1. Illustrates the number of children with in each age group.

A standard dose of 5 PPD units (0.1 mL) was injected intradermally (between the layers of dermis) and

read 48 to 72 hours later, Induration appeared in positive patient (Figure 2).



Figure 2: A patient with erythema (redness) in positive Mantoux test case.

Early morning gastric aspirate samples were collected from children under six 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 questionnaire was used to collect data about the patients. On obtaining the study results, the data were completed and analyzed statistically using χ^2 and p-value. The processing of the samples was carried out in a biosafety cabinet. Each sample was processed by the modified Petroff's method and subjected to Ziehl-Neelsen (ZN) staining, Fluorescent Auramine-O

(AO) staining and culture on modified Lowenstein-Jensen medium.

Phenotypic Characterization

Auramine-O Fluorochrom Staining Technique

Smears were flooded with freshly filtered auramine-O for 7-10 minutes, washed well with running water, then decolorized by covering with 0.5% acid-alcohol for 2 minutes, twice, washed well with running water and lastly stained with 0.1% potassium permanganate for 30 seconds as counter stain. Smears were examined under fluorescent microscope; the bacilli appeared as slender bright yellow fluorescent rods standing out clearly against a dark background (Figure3).

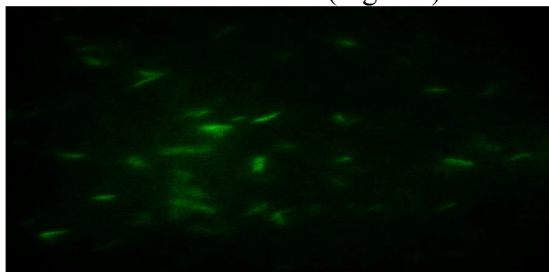


Figure 3: Shows bright green fluorescent rods of M. tuberculosis against a dark background.

Slide Smear for Ziehl-Neelsen Stain

Smears were fixed by gentle heat, and then flooded with carbol fuchsin and heated till steam raises, 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 were

examined using a light microscope scanning at least 100 oil immersion fields before reporting a smear as negative⁽⁷⁾

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 (Figure 4).

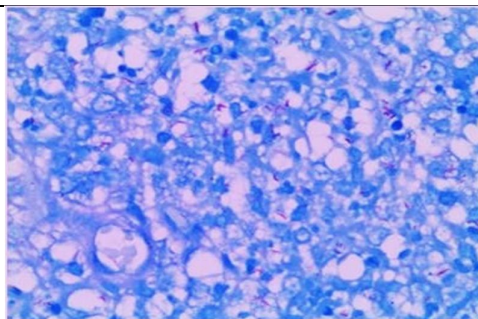


Figure 4: Appearance of *M. tuberculosis* when stained with ZN stain.

Culture

Lowenstein-Jensen (LJ) media were prepared. After processing, 0.25ml of the sediment from the decontaminated sputum specimen was inoculated on to the surface of two LJ media. After spreading the inoculum over the surface of the slant, the tubes were incubated at 37 °C and left in the

slanted position for two days to permit even distribution of the inoculum over the entire surface of the medium. The tubes were then placed upright and incubation continued for 8 weeks. A positive culture growth shows buff to yellow, rough wrinkled colonies (Figure 5). A negative report was given if no growth appeared after 8 weeks⁽⁸⁾.



Figure 5: Typical growth of *M. tuberculosis* on LJ medium.

RESULTS

Out of 197 patients, 86, 16, 22 and 32 cases were found to be positive for tuberculosis by TST, ZN staining, AO staining and culture techniques respectively. The positivity rate for TST, ZN staining, AO staining and culture in this study was 43.7%

(86/197), 8.1% (16/197), 11.2% (22/197) and 16.2% (32/197) respectively. The combined positivity using laboratory techniques (ZN, AO and culture) was 18.27% (36/197).

Table 1 show that scores are definitely higher by 86 TST positive as against 32 positive by culture method.

Table 1. Shows cross tabulation of culture and TST results with P-value.

		culture		Total	P- Value
		positive	negative		
TST	positive	32	54	86	0.00
	negative	0	111	111	
Total		32	165	197	

In comparison of each technique to culture as gold standard it showed, 50% (16/32) of ZN were found to be positive, 68.7% (22/32) were found to be positive from AO staining. On the other hand TST showed positive result more than culture technique. 37.2% (32/86) of culture were positive in comparison to TST test.

Correlation between TST and ZN, AO Stain and Culture

Disregarding the scores, 148 of 197 samples gave identical results. In other words, there was 75.1% agreement or 24.9% disagreement between TST and

other laboratory techniques. There were 127 (64.5%) agreement and 70 (35.5%) disagreement between ZN and TST. while 133 (67.5%) TST agree with auramine and 64 (32.5%) were disagree. In comparison, the agreement between TST and culture was the highest percentage 143 (72.6%) and 54 (27.4%) disagreement.

The difference between culture technique and other methods was found to be highly significant using Chi-Square test, p-value < 0.001. Tables 1, 2 and 3.

Table 2. Shows cross tabulation of culture and ZN results with P-value.

		culture		Total	P- Value
		positive	negative		
ZN	positive	14	2	16	0.00
	negative	18	163	181	
Total		32	165	197	

Table 3. Shows cross tabulation of culture and Auramine results with P-value.

		culture		Total	P- Value
		positive	negative		
Auramine	positive	19	3	22	0.00
	negative	13	162	175	
Total		32	165	197	

Sensitivity and specificity

The sensitivity and specificity of TST and microscopic techniques based on results of culture as gold standard technique for diagnosis of tuberculosis were as follow; sensitive were 100%, 59.38%, 43.75% for TST, Auramine, and ZN test respectively. While specificity were 67.27%, 98.18%, 89.79%. Positive Predictive Value (PPV) was 37.23% for TST, 86.36% for Auramine and 87.50% for ZN stain. Negative Predictive Value

(NPV) was 100%, 92.57% and 90.06% respectively.

DISCUSSION

In this study, we have shown that only 41.9% (36/86) of TST positive children were found to be positive with other laboratory tests (ZN, Ao stain and culture). Most of participants are already BCG-vaccinated, a factor known to affect the specificity of TST. The assumption that factors other than tuberculosis infection are widely contributing to positive TST reactions among children in Sudan is supported

by this study, as only 41.9% of TST positive children also had a positive with other laboratory tests. Thus, other laboratory tests seem to be more specific than TST. Due to the test's low specificity, most positive reactions in low-risk individuals are false-positives⁽⁹⁾. A false positive result may be caused by Non-tuberculous *Mycobacteria* (NTM) or previous administration of BCG vaccine. Prior vaccination with BCG may result in a false-positive result for many years afterwards⁽¹⁰⁾. TST reactions caused by previous BCG-vaccination or infections with NTM are expected to be moderate and in the range of 6–14 mm⁽¹¹⁻¹²⁾, consistent with our study where 43.7% of the positive TST reactions occurred within this range. The effect of infant BCG-vaccination on TST is perceived to be minimal, especially more than 10 years after vaccination⁽¹³⁾. Contrary to our results, other studies have reported a significantly better agreement between the tests for unvaccinated than vaccinated groups⁽¹⁴⁻¹⁵⁾. Different risk profile for tuberculosis exposure between the vaccinated and unvaccinated participants in our study may explain the lack of difference among the children. Infections from NTM could explain the high number of TST positive children.

CONCLUSION

In conclusion, TST is highly sensitive in detection of TB infection, but low specific in comparison with ZN, Auramin O and culture. The results indicate that factors other than tuberculosis, such as NTM infection or previous BCG-vaccination, are widely contributing to positive TST results among children in Sudan. Further studies are needed to confirm findings of the result study and to evaluate the TST for the diagnosis of tuberculosis in children.

REFERENCES

1. WHO–WHO/TUB., (2003). Guidelines for Tuberculosis Treatment in Adults and Children in National Tuberculosis Programs. Tuberculosis Unit Division of Communicable Diseases. Third edition. Geneva. *World Health Organization*.
2. Jennifer L and Mona R., (2009). Diagnosing Childhood Tuberculosis: Traditional and Innovative Modalities. *Curr Probl Pediatr Adolesc Health Care* 39:61-88.
3. CDC., (2013). www.cdc.gov/tb/topic/populations/TBInChildren/global.htm.
4. Health Protection Agency., (2012). www.hpa.org.uk/infections/topics_az/tb/menu.htm.
5. CDC., (2013). www.cdc.gov/nchstp/tb/faqs/qa.htm.
6. Health Protection Agency., (2012). www.hpa.org.uk/tbknowledge/default.htm.
7. Forbes BA, Sahm DF, Weissfeld AS., (1998). Role of Microscopy in the Diagnosis of Infectious Diseases. In: Bailey and Scotts' Diagnostic Microbiology. 10th edition. *Missouri: Mosby*; 134-49.
8. Cruickshank R, Duguid JP, Swain RHA, Marmion BP., (1975). *Mycobacterium*. In: Medical Microbiology - The Practice of Medical Microbiology. Vol.2. 12th ed. *Edinburgh: Churchill Livingstone*; p. 387-91.
9. Starke J (1996). "Tuberculosis Skin Testing: New Schools of Thought". *Journal of the American Academy of Pediatrics* 98: 123–25.
10. Chaturvedi N and Cockcroft A (1992). "Tuberculosis screening among health service employees: who needs chest X-rays?". *J Soc Occup Med* 42: 179–82.
11. Bahrmand AR, Madani H, Samar G, Khalilzadeh L, Bakayev VV, Yaghli M, (1996) Detection and identification of non-tuberculous

mycobacterial infections in 6,472 tuberculosis suspected patients. *Scand J Infect Dis.*;28:275–278.

12. Von Reyn CF, Horsburgh CR, Olivier KN, Barnes PF, Waddell R, Warren C (2001) Skin test reactions to *Mycobacterium tuberculosis* purified protein derivative and *Mycobacterium avium* sensitin among health care workers and medical students in the United States. *Int J Tuberc Lung Dis.*;5:1122–28.

13. Farhat M, Greenaway C, Pai M, Menzies D (2006). False-positive tuberculin skin tests: what is the absolute effect of BCG and non-

tuberculous mycobacteria? *Int J Tuberc Lung Dis.*;10:1192–204.

14. Brock I, Weldingh K, Lillebaek T, Follmann F, Andersen P.(2004) Comparison of tuberculin skin test and new specific blood test in tuberculosis contacts. *Am J Respir Crit Care Med.*;170:65–69.

15. Diel R, Ernst M, Doscher G, Visuri-Karbe L, Greinert U, Niemann S, (2006). Avoiding the effect of BCG vaccination in detecting *Mycobacterium tuberculosis* infection with a blood test. *Eur Respir J.*;28:16–23.