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Direct Detection of *Mycobacterium Tuberculosis in Sputum* by Polymerase Chain Reaction and Zieh-Neelsen Stain

KEYWORDS: Mycobacterium tuberculosis, PCR, Tuberculosis, ZN stain, Sudan.

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ABSTRACT *Background: Tuberculosis (TB) is a major public health threat, annually affecting new individuals worldwide, especially those in developing countries. Rapid, sensitive and specific technique for detection of the agent and effective treatment are two important factors in controlling this disease.*

Aim: The current study aimed to evaluate the sensitivity and specificity of direct and concentrated Zieh-Neelsen (ZN) stain in respect to Polymerase Chain Reaction (PCR) technique in the direct detection of Mycobacterium tuberculosis from clinical specimens

Methodology: This was a descriptive cross sectional study done on patients suspected of having tuberculosis attending Al-Shab Teaching Hospital; Khartoum-Sudan during October 2013. A total of 50 sputum specimens were examined by Polymerase Chain Reaction (PCR) and Zieh-Neelsen (ZN) Stain.

The Results: Mycobacterium tuberculosis was detected in 9 (18%) by direct smear ZN stain, 20 (40%) by concentrated ZN stain and 27 (54%) by PCR. The sensitivity and specificity of direct ZN smear in respect to PCR were 26% and 91% respectively; while the sensitivity and specificity of concentrated ZN smear in respect to PCR were 52% and 74%, respectively.

Conclusion: Although the high specificity of ZN smear, but it lacks the sensitivity. PCR is very useful for rapid detection of M. tuberculosis infections.

Introduction:
Tuberculosis (TB) remains a major global health problem. It causes ill-health among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV). The latest reports estimate that there were 8.6 million new TB cases in 2012 and 1.3 million TB deaths (just under 1.0 million among HIV-negative people and 0.3 million HIV-associated TB deaths). Most of these TB cases and deaths occur among men, but the burden of disease among women is also high. In 2012, there were an estimated 2.9 million cases and 410 000 TB deaths among women, as well as an estimated 530 000 cases and 74 000 deaths among children. The number of TB deaths is unacceptably large, given that most are preventable if people can access health care for a diagnosis and the right treatment is provided. Short-course regimens of first-line drugs that can cure around 90% of cases have been available for decades [(1)]. Sudan has a high burden of TB with a prevalence of 209 cases per 100,000 of the population and 50,000 incident cases during 2009 [(2)].

The conventional laboratory diagnosis of tuberculosis is based on the method of Ziehl-Neelsen acid–fast bacilli stain and culture of MTB [(3)]. The Ziehl-Neelsen stain is a rapid and cheap method, but it lacks sensitivity. The culture requires time and viable microorganisms, which are difficult to obtain, especially in treated patients. Several rapid methods for MTB diagnosis, such as DNA probes that require sophisticated equipment, have been developed [(4)]. The polymerase chain reaction (PCR) is an alternative method that can amplify a small fragment of DNA with high specificity and sensitivity for the diagnosis of infectious diseases [(5)]. PCR has recently been used to detect MTB in respiratory and other samples [(6,7)].

Methods
Specimens: A total of 50 sputum specimens from patients with signs and symptoms of pulmonary TB attending Al-Shab Teaching Hospital during October 2013, were submitted for AFB and PCR.

Ziehl-Neelsen stain: A direct and concentrated smear were made from each sputum specimen and stained by the ZN method according to Clinical and Laboratory Standards Institute (CLSI Guidelines for Mycobacteriology 2013) [(8)].

Digestion and Decontamination of sputum samples for PCR: Sputum samples were digested in a 10 ml solution containing 4% Sodium hydroxide with 2.9% sodium citrate buffer in a 50 ml falcon tube for 15 minutes. The homogenization of the sputum was done using a vortex mixture placed in Biosafety Cabinet Class II. The reaction was stopped by the addition of a phosphate buffer to a final volume of 50 ml. This was followed by centrifugation at 3500 X g for 15 minutes. The suspension was discarded, leaving 2 ml to resuspend the pellet. Prelysis buffer was added to the pellet and then heat killed at 65°C for 15 minutes ready for DNA preparation.

DNA Extraction: The DNA was extracted from the sputum pellet suspension using DNG™-Plus – MTB DNA Extraction Solution (Catalog No: DN8118C); Cinna Gene; Tehran – Iran.

Polymerase Chain Reaction (PCR): The IS6110 gene coding for Mycobacterium tuberculosis was detected by PCR, according to the protocol described by Cinna Gene MTB PCR Detection kit; Tehran-Iran; (Cat. No.: PR7935C). The primer sequences were as follows: forward primer P43 5′ TCA GCC GCG TCC ACG CCGCCA 3′; reverse primer P53 5′ CCG ACC GCT CCG ACC GAC GGT 3′. The reaction was performed in a 25 µl volume. Thermo-cycling conditions in a Techne thermocycler (Bibby Scientific Limited, Beacon Road, Stone, Staffordshire ST15 0SA, UK) were as follows: 93°C for 60 seconds, followed by 37 cycles of 93°C for 60 seconds, 72°C for 20 seconds and 72°C for 30 seconds, with a final extension at 72°C for 2 minutes.

The amplified products 10 µl were separated by electrophoresis on 2% agarose gel and visualized by staining with ethidium bromide using UV gel documentation system. A 163-bp PCR product was amplified with the above IS6110 gene specific primers.

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Ethical Clearance:
Approval was taken by the ethical review board of the Faculty of Medical Laboratory Sciences Al-Neelain University. Verbal consent was taken from each study unit.

The Results:
Demographic characteristics of these patients are listed in table (1). Male represented 70%, while female represented 30%. The age range between 2 and 75 years and the majority of them were middle age (between 16 and 45 years). Regarding the signs and symptoms of tuberculosis, the majority of patients (72%) represented with chronic cough, while 64% suffering from night sweat.

Table (1) Patients demographic characteristics and clinical signs and symptoms

	Number	%
Gender		
Male	35	70
Female	15	30
Age groups		
01 - 15 Year	5	10
16 - 30 Year	15	30
31 - 45 Year	15	30
46 - 60 Year	9	18
61 - 75 Year	6	12
Signs and symptoms		
Chronic cough	36	72
Haemoptysis	28	56
Night sweat	24	48
Weight loss	32	64
Fever	28	56

As shown on the table (2) Mycobacterium tuberculosis was detected in 9 (18%) by direct smear ZN stain, 20 (40%) by concentrated ZN stain and 27 (54%) by PCR.

Table (2) Detection of Mycobacterium tuberculosis

	Direct ZN		Concentrated ZN		PCR	
	No.	%	No.	%	No.	%
Positive	9	18	20	40	27	54
Negative	41	82	30	60	23	46

The sensitivity and specificity of direct ZN smear and concentrated ZN smear were evaluated against PCR as standard test (Table 3). The sensitivity and specificity of direct ZN smear were 26% and 91%, respectively; while the sensitivity and specificity of concentrated ZN smear were 52% and 74% respectively.

Table (3) Correlation between PCR and direct and concentrated ZN stain

	PCR Positive	PCR Negative	Total
Direct ZN Positive	07	02	09
Direct ZN Negative	20	21	41
Total	27	23	50
Concentrated ZN Positive	14	6	20
Concentrated ZN Negative	13	17	30
Total	27	23	50

Discussion:
Tuberculosis is a major public health problem, annually affecting new individuals, especially in developing countries. Worldwide, the main problems in TB management remain the early diagnosis of MTB and drug-resistance testing. Rapid diagnosis and appropriate chemotherapy become the first priorities and a serious challenge in the improvement of TB treatment and the reduction of the dissemination of MTB strains [1]. Currently, TB screening is done by conventional techniques, including the method of Ziehl-Neelsen staining and MTB culture. However, recent studies have shown that up to half of the new cases are smear negatives when diagnosed by ZN stain [4]. Unfortunately, the Ziehl-Neelsen staining lacks sensitivity, and bacteria culture is time-consuming [5].

There is a clear need to implement reliable molecular techniques for the detection of all forms of TB strains to improve the management of TB. Molecular techniques are rapid and prevent crucial delays. In the last decade, advances in molecular biology have made it possible to use rapid and specific techniques to detect MTB DNA in samples. It is widely accepted that PCR is a new and rapid technique for the diagnosis of bacterial DNA with high sensitivity and specificity, even in patients who have undergone antibiotic treatment because the DNA is still detectable in the absence of a viable microorganism [9].

Direct ZN smear can detect the organism, but for patients who have a small number of organisms, the smear loses sensitivity. In our study, from 27 PCR positive samples only 7 (26%) were positive by direct ZN smear, and 14 (52%) were positive by concentrated ZN smear. On the other hand direct ZN smear showed high specificity (91%) comparing with concentrated ZN smear (74%).

In our study, there are 2 samples positive by direct ZN smear and 6 samples positive by concentrated ZN smear, but they were negative by PCR, this discrepant results of ZN smears and PCR were attributed to the presence of inhibitors during sample preparation. A proper sample preparation for PCR to eliminate inhibitors is a necessary step.

A Cinna pureTM DNA extraction kit that produced the DNA within 15 minutes was used. This greatly solved the problem of the lengthy procedures associated with other extraction methods such as the phenol and Chelex extraction methods that may take between 2 - 3 days. The ability of this kit to produce high yields of DNA within 15 minutes was demonstrated and this facilitated the PCR evaluation process. Studies have demonstrated that the extraction method employed and the quality of the DNA used in PCR has direct effects on the sensitivity and specificity of PCR.

In conclusion, a molecular approach, based on the amplification of IS6110 gene by PCR, is a reliable and rapid method that could be used to detect MTB strains in clinical specimens.

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