Detection of *Mycobacterium tuberculosis* in sputum using conventional methods and PCR

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**Abstract**

We evaluated the Polymerase Chain Reaction (PCR) based test for the detection of *Mycobacterium tuberculosis* complex, the common deadly infectious disease, and conventional methods smear stained with acid fast staining and cultures on Lowenstain–Jensen medium in 50 sputum samples which were collected from patients with symptoms of tuberculosis in Nineveh governorate.

The result indicated that, 19 (38%) were smear positive by ZN stain direct or traditional method, and 20 (40%) were smear positive by ZN stain concentrated method, 17 (34%) were smear positive by Kinyoun’s method, 21 (42%) were LJ culture positive for *M. tuberculosis*, and 22 (44%) were given specific bands of amplified DNA in a agarose (2%) with lengthed specific fragment is 390bp and internal control 750 bp using MTB complex 390\ 750 IC from sacace biotechnologies kit. This test is a valuable diagnostic tool for today’s mycobacteriology laboratory.
Introduction

Tuberculosis (TB for tubercule bacilli) is a common and deadly infectious disease caused by *Mycobacterium tuberculosis*. (Godreuil et al., 2007)

*Mycobacterium tuberculosis* is strongly acid fast, aerobic rods, possesses a complex lipid rich cell wall, this cell wall is responsible for many of the characteristic properties of the bacteria (eg acid fastness, slow growth it divides every 12-24 hours, resistance to detergents, resistance to common antibiotics, antigenicity, clumping) (Talaro and Talaro, 1999; Murray et al., 2005).

World health organization (WHO) estimated that one third of population has been infected with TB and new infections occur at a rate of 1 per second, and 8 million new tuberculosis cases occur each year. Moreover nearly 3 million people die annually of tuberculosis (WHO, 2009). Regions with the highest incidence of disease were southeast asia, sub-Saharan Africa, and the eastern Europe. (Murray et al., 2005)

Iraq is one of the countries in WHO Eastern Mediterranean region (WHO-EMR) with a relatively high TB incidence rate (56/100,000) and low case detection rate (45%) (World Health Organization, 2009). (Merza et al., 2011).

Direct microscopy, culture on Lowenstein–Jensen (LJ) medium and biochemical tests for detecting and identifying members of the *M.tuberculosis* complex (MTBC) are still used in mycobacteriology laboratories. Sensitivity using microscopy is poor (of the order of 105 acid-fast bacilli/mL sputum) and culture methods require 3 to 8 weeks for completion. This merely indicates evidence of mycobacteria and additional biochemical testing is undertaken to identify the species, in itself a time consuming and challenging task necessitating experienced personnel. (Abu-Amero and Halablab, 2004; Hashim et al., 2007).
The demand for sensitivity, specificity and speed of *M. tuberculosis* detection led to the development of nucleic acid based amplification tests to target mycobacterial DNA or RNA directly from the clinical samples. (Hawkey, 1994)

Among nucleic acid-based techniques for the diagnosis of TB, the PCR is the most widely published amplification technique. An increasing number of laboratories are using the PCR technique to detect TB in clinical samples since it provides good rates of positive results and faster turnaround times than culturing (Rau and Libman, 1999)

Early diagnosis of tuberculosis makes effective treatment possible and increases the probability of clinical outcome owing to quite effective anti-tuberculosis therapy, however the tuberculosis diagnosis has certain difficulties. According to international standards, tuberculosis diagnosis must be confirmed either by bacteriology or by histology studies but the bacteriological methods do not always allow to detect its in people affected with pulmonary tuberculosis and especially with extrapulmonary tuberculosis (Broccolo *et al.*, 2003; Saleh *et al.*, 2007).

The application of molecular biology methods allow to overcome the difficulties in the diagnosis of *M. tuberculosis* but due to the biological peculiarities of this microorganism and immune response of human organism, tuberculosis can not be diagnosed by one method. (Narayanan, 2004)

Direct and indirect diagnostic methods are applied in phthisiology. Smear bacteriscopy with ziehl neelsen stain techniques is a rapid and cheap method, but it has low sensitivity, not high specificity, and cannot differentiate TB from non-tuberculous mycobacteria. The diagnostic sensitivity of the method doesn't exceed 20 - 40%. Culture have high sensitivity and specificity however due to a slow growing tendency it takes 2 – 12 weeks to get a result. The indirect method such as X – ray diagnostics, tuberculin diagnostics, detection of tuberculosis antibodies do not directly identify TB, however, they give an
understanding of current changes in organs (Supply et al., 2001).

MTB complex 390\750 IC is an in vitro nucleic acid amplification test for qualitative detection of *Mycobacterium tuberculosis* in the sputum, urine, bronchial lavages, tissues, and other biological materials. It is based on 3 major processes: sample preparation, nucleic acid amplification of DNA using specific *M. Tuberculosis* primers, and detection of the amplified product on agarose gel. The kit contains the internal control which may be used in the isolation of procedure and serves as amplification control for each individually processed specimen and to identify possible inhibition. So, the objective of this study was to evaluate the MTB complex 390\750 IC for the detection of MTBC in sputum samples and compared to those obtained using conventional LJ culture medium and acid fast staining.

### Materials and Methods

#### 1- Sample collection:

50 sputum samples were collected by using sterile container for bacteriological study. All samples were collected from two gender and all ages of patients with clinical signs or symptoms of pulmonary or extrapulmonary TB, in Alkamalia, Ibn Sena, Alsalam, Ibn Alatheer general hospitals and consultant clinic for chest and respiratory diseases in Nineveh governorate. The collection of samples was carried out from September 2010 to September 2011.

#### 2- Processing of Samples:

The sputum specimens were digested and decontaminated by using Sodium hydroxide procedure (Petroff).

An equal volume of sputum was mixed with 4% sodium hydroxide (previously sterilized by autoclaving) in sterile centrifuge tube. The mixture was allowed to stand at room temperature for 15 minutes with occasional gentle shaking. And the mixture centrifuged at 3000 rpm for 15 minutes. The resultant supernatant was discarded and the sediment neutralized by distilled water or by drop with a 2 mol /
1N HCl solution containing 2% of phenol red combined with shaking until the colour changes from red to yellow. And the sediment used for preparing the concentrated smear method and inoculating culture media. (Vandepitt et al., 2003; Mankhi, 2010).

3- Smears stained with Acid Fast Bacilli staining:

Three smears were prepared from all 50 sputum samples, the first Fixed smear were prepared from sputum and stained with Ziehl Neelsen (ZN) staining for direct or traditional method according to (Winn et al., 2006). The Second fixed smear were prepared from sediment of processed sputum and stained with ZN staining for concentrated method. The third fixed smears were prepared from sputum and stained with Kinyouns method according to (Winn et al., 2006). And all smears were examined with 100x oil immersion objectives using light microscopy.

4- Mycobacterial Culture:

Lowenstein Jensen (LJ) medium was prepared by method described by (Mankhi, 2010). The LJ medium slants were inoculated with 1ml of processing sputum as in paragraph 2 and the inoculated slants were kept in incubator at 37 for (4-8) weeks.

The growth of M. tuberculosis thus obtained was identified by their rate of growth, colonial morphology, Ziehl Neelsen staining and biochemical tests, five biochemical tests were used in this study which include: niacin accumulation test, pyrazinamidase test, nitrate reduction test, tween 80 hydrolysis test, urase test and growth on LJ agar containing 5% NaCl according to (Winn et al., 2006).

5- Molecular Methods:

DNA extraction procedure from positive acid fast stain:

Sputum was collected into 50ml. single use PP tubes with screw cap. In a biological safety cabinet, homogenize samples after mixing with equil volume of 4% NaOH solution (N-acetyl−L-cysteine may be added if required in
the amount of 50 – 70 mg per sample ). mix intensely with tube rotator for 5 – 20 minutes ( depending on the density of the sample). Samples were centrifuged at 3000 rpm ( 2800 - 3000 g ) for 15 min and carefully discard the supernatant leaving 500 – 1000 μl in the tube. resuspended sediment and transfer it into a 1.5 ml tube. Samples were centrifuged at 12000 rpm for 5 – 10 min, the supernatant was discarded and the same tube was used for DNA isolation from sample sediment. 100 μl of the solution used for DNA extraction by using sacace DNA extraction kit K2- 9 and then 10 μl of extracted DNA used ( from processed sputum and colonies of *M. tuberculosis* culture ) for PCR- mix- 1 tubes were prepared and then transfer them into thermocycler, only when temperature reached 95 ºC and start the program which presented in table 1:

Table 1 : Thermocycler with block temperature adjustment : MiniCycler , PTC – 100 ( MJ Research )

<table>
<thead>
<tr>
<th>Step</th>
<th>ºC</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95 ºC</td>
<td>Pause</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>95 ºC</td>
<td>15 min</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>95 ºC</td>
<td>20 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70 ºC</td>
<td>20 sec</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>72 ºC</td>
<td>20 sec</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>72 ºC</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>10 ºC</td>
<td>Storage</td>
<td></td>
</tr>
</tbody>
</table>

6- **Gel Electrophoresis**:  
2% agarose was prepared by adding 2 g of agarose to 100 ml of 50X TAE buffer and then it was solubilized by heating in boiling water path. The agarose was left to cool at 50 – 60 ºC before adding the ethidium bromide and pouring the gel. 10 μl of ethidium
bromide ( 10 mg \ ml ) was added to the agarose.

**Results and Discussion**

From a total of 50 sputum sample tested, 19 (38%) were smear positive by ZN stain direct or traditional method, and 20 (40%) were smear positive by ZN stain concentrated method, 17 (34%) were smear positive by Kinyoun's method, 21 (42%) were LJ culture positive for *M. tuberculosis* and 22 (44%) were PCR positive as shown in (table 2).

*(Table 2)* The result of conventional and PCR methods.

<table>
<thead>
<tr>
<th>Methods</th>
<th>No. sample tested</th>
<th>Positive sample</th>
<th>Negative sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number</td>
<td>%</td>
</tr>
<tr>
<td>1- Acid fast stained</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smear :</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A- Ziehl Neelsen Stain</td>
<td>50</td>
<td>19</td>
<td>38%</td>
</tr>
<tr>
<td>Direct smear</td>
<td>50</td>
<td>20</td>
<td>40%</td>
</tr>
<tr>
<td>Concentrated Method</td>
<td>50</td>
<td>17</td>
<td>34%</td>
</tr>
<tr>
<td>B- Kinyoun's Method</td>
<td>50</td>
<td>21</td>
<td>42%</td>
</tr>
<tr>
<td>2- Mycobacterial culture on</td>
<td>50</td>
<td>22</td>
<td>44%</td>
</tr>
<tr>
<td>LJ medium</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3- Polymerase Chain Reaction</td>
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</tbody>
</table>
The positive cultured on LJ slant for 4-8 weeks, the appearance of colonies were slow growers waxy rough, tough, and buff coloured colonies against the green egg based medium as in (Figure 1).

(Figure 1) Growth of M. tuberculosis on LJ agar slant after 8 weeks of incubation at 37 °C. Colonies are rough, tough, and buff coloured colonies.

(Figure 2) show smear of M. tuberculosis grown on LJ medium stained with ZN stain showing the arrangement of bacilli in bundles and cord formation. Cord formation due to the high wax content of the cell wall, these rods tend to adhere to each other after cell division to form cords. (Alexander and Strete, 2001). Acid fast bacilli appear red rods.

(Figure 2) Smear of M. tuberculosis prepared from LJ culture medium stained with Ziehl Neelsen stain showing arrangement of bacilli in bundles and cord formation. Acid fast bacilli appear red rods. (ZN stain X1000).
All strains grown on LJ slants were identified as *M. tuberculosis* by their rate of growth, colonial morphology, ZN staining, and biochemical tests; these were: pyrazinamidase test, niacin accumulation test, nitrate reduction test, tween 80 hydrolysis test, urease test and growth on LJ slants containing 5% NaCl. The results representing in (table 3).

**Biochemical tests for *M. tuberculosis***.

<table>
<thead>
<tr>
<th>Biochemical test</th>
<th>Biochemical characteristics of <em>M. tuberculosis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrazinamidase test (4 days)</td>
<td>+ band of red colour</td>
</tr>
<tr>
<td>Niacin accumulation test</td>
<td>+ yellow in colour</td>
</tr>
<tr>
<td>Nitrate reduction test</td>
<td>+ red colour</td>
</tr>
<tr>
<td>Tween 80 hydrolysis test (5 days)</td>
<td>_ no red colour</td>
</tr>
<tr>
<td>Urease test</td>
<td>+ pink colour</td>
</tr>
<tr>
<td>Growth on LJ slants containing 5% NaCl (4 weeks)</td>
<td>_ no growth</td>
</tr>
</tbody>
</table>

(Figure 3) Show two tubes of Dubos base, the right containing pyrazinamide and the left negative control devoid of reagent. Both tubes had previously been inoculated with a substrate of a strain of *M. tuberculosis*. The development of pink-red band at the reagent layer toward the surface of the agar in the right tube, after addition of ferrous ammonium sulfate reagent, indicates a positive test and the ability of this strain to deaminate pyrazinamidase. (Winn et al., 2006).
The result of this study is consistent with (Essa, 2001) that is ZN stain concentrated method is more sensitive than ZN stain direct method in detecting *M. tuberculosis* because the liquefaction, digestion and concentrated method of sputum facilitate to distinguish acid fast bacilli in microscopic field.

(Winn et al., 2006) indicated that is ZN stain more sensitive than Kinyouns method in detecting lighten staining organisms. In Kinyouns method *M. tuberculosis* appears as slender red rods. The classic ZN stain requires heating the slide for better penetration of stain into mycobacterial cell wall hence it is also known as ahot stain. Kinyouns acid fast stain is similar to ZN stain but without heat and called cold stain. (Fobes et al., 2002).

The study also indicated that culture of clinical specimen is more sensitive than acid fast smear and this is consistent with (Aftab et al., 2008; Al Saqr et al., 2009) because culture detects tuberculosis cases earlier before they become infectious since culture techniques can detect few bacilli (only 10-100 viable organisms will result in a positive culture while a minimum of 5000-10000 acid fast bacilli per ml are required for detection by smear)(Murray et al., 2005; Robledo et al., 2006). In addition to that isolates obtained from cultures can be used for *mycobacterial* species identification, determination of drug susceptibility test and molecular epidemiology. Positive
cultures of *M. tuberculosis* are used to confirm the diagnosis of TB. (Small et al., 1994; Ani, 2008).

Result of the conventional methods were confirmed by molecular methods, the PCR it give accurate positive result. Chromosomal DNA samples were resolved by horizontal agarose gel electrophoresis, the amplified DNA bands appear at 390 bp showed in figure 4:

![Figure 4](image)

Figure 4: P: positive control, N: negative control, S1, S2, S3, S4: PCR amplification results of *M. tuberculosis* appeared at 390 bp. The amplified fragments were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide at 100 volts for 60 min. photographed under UV light.

Although molecular techniques have been widely evaluated in diagnosis of TB. The PCR should be particularly useful for confirmed the result of the conventional methods which biochemical and growth data are difficult to obtain. The PCR give high sensitivity and specificity for the *Mycobacterium*
Detection of Mycobacterium tuberculosis (Abu-Amero and Halablab, 2004; Negi et al., 2005; Al saqur et al., 2009). The PCR method is more accurate and faster than conventional method for TB diagnosis (Janata et al., 2000; Moreira-Oliveira et al., 2008). Early diagnosis of TB disease is crucial in initiating treatment and interrupting the strain transmission. Rapid diagnosis will prevent the development of drug resistant M. tuberculosis bacteria. (Bruchfeld et al., 2002; Yam, 2006; Dou et al., 2008; Merza et al, 2011).

Recommendation

1- We must use molecular method (PCR) in the hospital laboratories of Nineveh governorate because it is faster than conventional methods, for early diagnosis of TB to initiate treatment and prevent strain transmission and prevent the development of multidrug resistant strain of M. tuberculosis.

2- Concentrate on a high risk group for having this infection like children <5 years and productive age group for an early diagnosis and treatment.

3- Using mass media for health education in our locality and adopting the preventive measures in eradicating TB.

4- Health services for diagnosis and treatment of TB cases should be available and accessible for all patients especially those in rural areas.

References


raphic Atlas for the Laboratory " . Benjamin Cummings, an imprint to Addison Wesley Longman, pp. 15-16.


Robledo, J.A.; Mejia, G.I.; Morcillo, N.; Chacon, L.; Camacho, M.; Luna, J.; Zurita, J.; Bodon, A.;


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the detection of Mycobacterium tuberculosis in chest samples by the traditional and PCR techniques. The study was conducted on 50 cases collected from the Ninawi Governorate.

The results showed that 19 (38%) were positive by the direct method of Zielenski staining, 20 (40%) were positive by the concentrated method of Zielenski staining, and 17 (34%) were positive by the method of Kinyoun and 21 (42%) were positive by the growth on Löwenstein-Jensen medium. PCR was used to amplify the 750 bp IC and 390 bp E. coli genes, and the extraction was done using a ready-to-use kit from the Italian company sacace.

The PCR test is considered an important diagnostic tool in modern laboratories for the study of Mycobacterium tuberculosis.