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

Kawkab Adris Mahmod, Basima Ahmad Abdulla*

Department of Anatomy
*Department of Biology
College of Medicine , University of
Mosul , Mosul , Iraq

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 Kinyouns method , 21 (42%) were LJ culture positive for *M. tuberculosis* **,** and 22 (44%) were given specific bands of amplified DNA in a garose (2%) with lengthed specific fragment is 390bpand 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 .

Basimaaa 138@yahoo.com

Introduction

Tuberculosis (TB for tubercule bacilli) is acommon 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 12-24hours , 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 1per second , and 8 million new tuberculosis cases occur each year . Moreover nearly 3million 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 lowenstain – 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 available for the diagnosis of TB , the PCR is the most widely published amplification technique . An increaseing 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 a 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 weekes 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 3major processes sample preparation,nucleic acid amplification of DNA using specific *M. Tuberculosis* primers and detection of the amplified product 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 specimens 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 :

50sputum 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- Processsing of Samples :

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

An equal volumes of sputum were 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 :

Lowenstain 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 inocubator 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 Mehods :

DNA extraction procedure from positive acid fast stain :

Sputum was collected into 50ml. single use PP tubes with screw cap. In a biological safley 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 centr-ifuged 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)

Step	**ºC**	**Time**	**Cycles**
1	**95 ºC**	**Pause**	
2	**95 ºC**	**15 min**	**1**
3	**95 ºC**	**20 sec**	**42**
	70 ºC	**20 sec**	
	72 ºC	**20 sec**	
4	**72 ºC**	**2 min**	**1**
5	**10 ºC**	**Storage**	

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

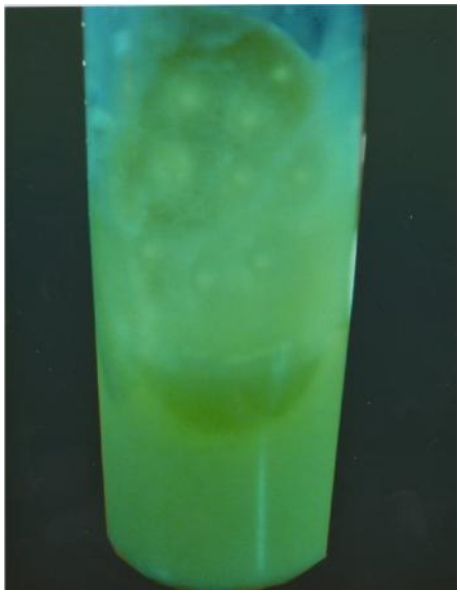
From a total of 50 sputum sample tested , 19 (38%) were smear positive by ZN stain direct or traditional method , and 20 (40%)were smear positive by ZN stain concentrated method , 17 (34%) were smear positive by Kinyouns method , 21 (42%) were LJ culture positive for *M. tuberculosis* and 22 (44%) were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

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

Methods	**No. sample tested**	**Positive sample**		**Negative sample**	
		Number	**%**	**Number**	**%**
1- Acid fast stained Smear :					
A- Ziehl Neelsen Stain :	50	19	38%	31	62%
	50	20	40%	30	60%
Direct smear	50	17	34%	33	66%
Concentrated Method					
B- Kinyouns Method	50	21	42%	29	58%
2- Mycobacterial culture on LJ medium	50	22	44%	28	56%
3- Polymerase Chain					

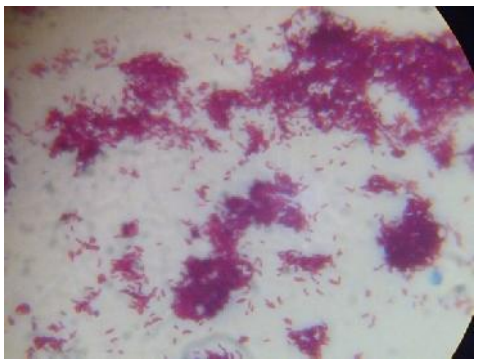
Reaction					

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 ºC . 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 bondles 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).

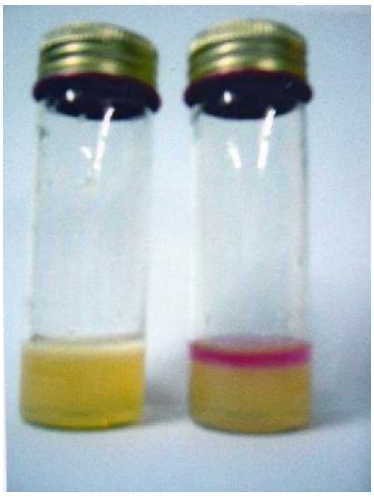
All strains grown on LJ slants were identified as *M.tuberculosis* by there rate of growth, colonial morphology , ZN staining, and biochemical tests these were : pyrazinamidase test , niacin accumulation test , nitrate reduction test , tween 80 hydrolysis test , urase test and growth on LJ slants containing 5% Nacl . The results representing in (table 3) .

(Table 3) Biochemical tests for *M . tuberculosis* .

Biochemical test	**Biochemical characteristics of *M. tuberculosis***
Pyrazinamidase test (4 days)	+ band of red colour
Niacin accumulation test	+ yellow in colour
Nitrate reduction test	+ red colour
Tween 80 hydrolysis test (5 days)	_ no red colour
Urease test	+ pink colour
Growth on LJ slants containing 5% Nacl (4 weeks)	_ no growth

(Figure 3) Show two tubes of Dubos base, the right containing pyraz-

inamide 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) .



(Figure 3) Pyrazinamidase test,right positive, left negative for *M.tuberculosis* .

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 Saqur *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 :

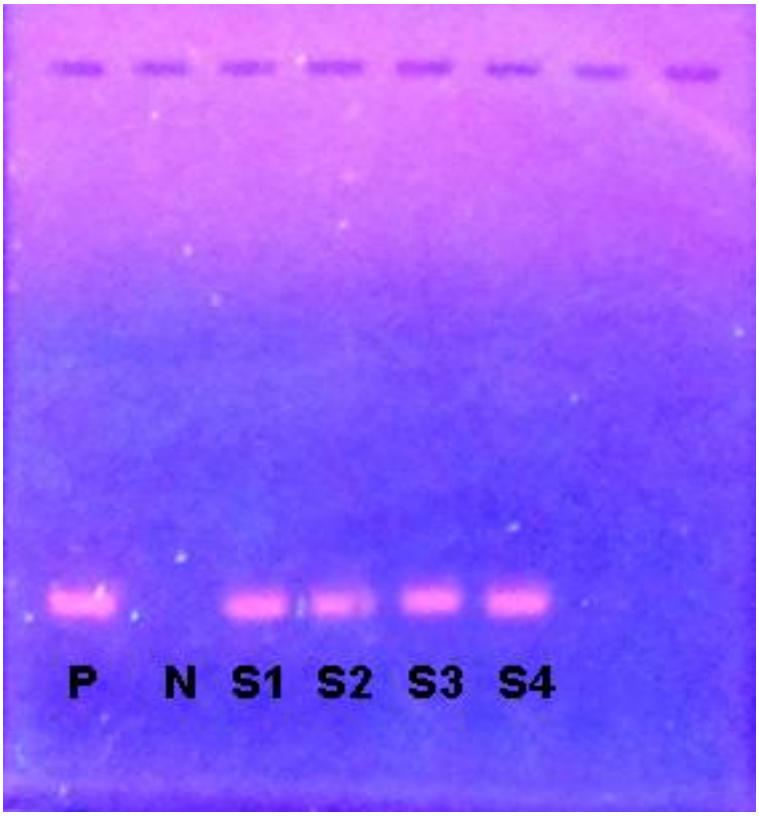


Figure 4 : P: positive control, N: negative control , S1,S2,S3,S4: PCR amplification results of *M. tuberculosis* appeared at 390 bp . The amplified fragments were

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 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 .

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التحري عن عصيات التدرن (السل) في القشع بالطرائق التقليديه وتقنية تفاعل البلمره المتسلسل

كوكب ادريس محمود, باسمة احمد عبد الله*

فرع التشريح ، كلية الطب

*قسم علوم الحياة /كلية العلوم

جامعة الموصل ، موصل ، العراق

الخلاصة

تقييم اختبار تفاعل البلمره المتسلسل للتحري عن عصيات التدرن (السل) الذي يعد من الامراض المعديه والمميته الذي تسببه عصسات التدرن *Mycobacterium tuberculosis* والطرق التقليديه المسحه المصبوغه بالصبغات الصامده للحامض والزرع على وسط لونيشتين جنسن للتحري عن العصيات في 50 عينة قشع جمعت من المرضى الذين تبدو عليهم اعراض التدرن في محافظة نينوى .

اوضحت النتائج ان19(38%) كانت موجبة بالطريقة المباشرة لصبغة زيل نلسن و 20(40%) كانت موجبة بالطريقة المركزة لصبغة زيل نلسن و17(34%) كانت موجبة بطريقة كينيون و 21 (42%) كانت موجبه الزرع على وسط لونيشتن جنسن وان22 (44%) اعطوا حزم خاصه للحامض النووي المضخم في الاكاروز (2%) وبطول 390 pb وسيطره داخلية IC 750pb وذلك باستعمال عدة عمل جاهزه للتضخم من شركة sacace الايطاليه . وهكذا فان اختبار PCR يعد من الادوات التشخيصيه الهامه في المختبرات المعاصرة المتخصصه بعلم عصيات التدرن .